e.2 HOSPITAL LIBRARY Nurses' Library

VOL. 32 JANUARY, 1947 No. 1

The Journal of Laboratory and Clinical Medicine

(DUPLICATE)

The Official Publication of the Central Society for Clinical Research

Clinical and Experimental

Announcement ---------- 1

Metabolic Function of Pteroylglutamic Acid and Its Hexaglutamyl Conjugate. I. Hematologic and Urinary Excretion Studies on Patients With Macrocytic Anemia. Frank H. Bethell, M.D., Muriel C. Meyers, M.D., Gould A. Andrews, M.D., Ann Arbor, Mich., and Marian E. Swendseid, Ph.D., Orson D. Bird, Ph.D., and Raymond A. Brown, Ph.D., Detroit, Mich. ---------- 3

Metabolic Function of Pteroylglutamic Acid and Its Hexaglutamyl Conjugate. II. Urinary Excretion Studies on Normal Persons. Effect of a Conjugase Inhibitor. Marian E. Swendseid, Ph.D., Orson D. Bird, Ph.D., Raymond A. Brown, Ph.D., Detroit, Mich., and Frank H. Bethell, M.D., Ann Arbor, Mich. 23

The Effect of the Methylated Xanthines on the Clotting Time of the Blood. N. C. Gilbert, M.D., Frederick Dey, M.D., and Ruth Trump, B.S., Chicago, Ill. 28

Penicillin in Beeswax and Peanut Oil, a New Preparation Which Is Fluid at Room Temperature: Absorption and Therapeutic Use. Harold L. Hirsh, M.D., Harry F. Dowling, M.D., Jean J. Vivino, M.D., and Georgine Rotman-Kavka, M.D., Washington, D. C. ---------- 34

Pharmacodynamic Effect in Man of Streptomycin Containing a Histamine-Like Factor. William L. Hewitt, M.D., and John J. Curry, M.D., Boston, Mass. 42

Histamine Antagonists. VI. Comparative Antihistaminic Activity of Some Ethylenediamine Drugs in the Guinea Pig. Sidney Friedlaender, M.D., Detroit, Mich., and Samuel M. Feinberg, M.D., and Alan R. Feinberg, M.D., Chicago, Ill. ---------- 47

Unusual Instances of Auricular Flutter. S. F. Aronson, M.D., and L. N. Katz, M.D., Chicago, Ill. ---------- 51

Effect of Oxygen on Malaria. R. H. Rigdon and Nona B. Varnadoe, Little Rock, Ark. ---------- 57

(Contents continued on inside cover)

PUBLISHED BY THE C. V. MOSBY COMPANY, 3207 WASHINGTON BLVD., ST. LOUIS 3
Copyright 1947 by The C. V. Mosby Company

Reprint

Determination of Normal Mean Corpuscular Weight (Erythrocyte). Chenting Ch'in, M.B., Ch.B., New York, N. Y. ---- 66

The Bone Marrow of Normal Cats. Arthur Sawitsky, M.D., and Leo M. Meyer, M.D., New York, N. Y. ---- 70

Laboratory Methods

Reactions of Various Parasitic Organisms in Tissues to the Bauer, Feulgen, Gram, and Gram-Weigert Methods. R. D. Lillie, Bethesda, Md. ---- 76

A Simple Method of Staining Malaria Parasites. George Joannides, M.D., Athens, Greece ---- 89

The Use of Russell Viper Venom as a Means of Following the Anticoagulant Action of 3,3'-Methylenebis (4-Hydroxycoumarin) in the Dog. Edwin J. de Beer, Tuckahoe, N. Y. With the Technical Assistance of Rocco Fanelli 90

Microanalysis of Opiates by X-ray Diffraction. S. T. Gross, Urbana, Ill., and Fred W. Oberst, Lexington, Ky. ---- 94

A Method of Bone Marrow Biopsy From the Rat. I. Myron Vigran, M.D., Rochester, Minn. ---- 102

The Use of Dried Antigen Preparations for Testing Pneumococcus Typing Sera. Janet M. Bourn, Ph.D., and Charmion M. Carnes, A. B. ---- 104

Book Reviews

Book Reviews ---- 107

Editor

CARL V. MOORE, M.D.
Washington University School of Medicine
600 South Kingshighway
St. Louis 10, Mo.

Board of Editors

EDGAR V. ALLEN, M.D.
Mayo Clinic, Rochester, Minn.

M. A. BLANKENHORN, M.D.
University of Cincinnati, Cincinnati

GEORGE E. BURCH, Jr., M.D.
Tulane University, New Orleans

PAUL R. CANNON, M.D.
University of Chicago, Chicago

JEROME E. CONN, M.D.
University of Michigan, Ann Arbor

CHARLES A. DOAN, M.D.
Ohio State University, Columbus

EDGAR S. GORDON, M.D.
University of Wisconsin, Madison

IRVINE H. PAGE, M.D.
Cleveland Clinic, Cleveland

ARMAND J. QUICK, M.D.
Marquette University, Milwaukee

WESLEY W. SPINK, M.D.
University of Minnesota, Minneapolis

CECIL J. WATSON, Ph.D., M.D.
University of Minnesota, Minneapolis

ARNOLD D. WELCH, Ph.D., M.D.
Western Reserve University, Cleveland

W. BARRY WOOD, Jr., M.D.
Washington University, St. Louis

Medical
Copy 2

ANNOUNCEMENT

The Central Society for Clinical Research and The C. V. Mosby Company have entered into an agreement which provides that THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE will be published as the official publication of the Central Society for Clinical Research. Beginning with Volume 32, this issue, the Central Society for Clinical Research will assume editorial direction of the JOURNAL.

The Board of Editors will consider for publication manuscripts which record original contributions in the broad fields of clinical investigation and clinical pathology. A "Methods" section will be retained. The works of *all authors* are invited; society affiliations will not prejudice or influence editorial decisions. The current practice of providing prompt publication of articles accepted by the Board of Editors will be continued.

Many of the papers which will appear in the first three issues were approved by the former editorial board.

METABOLIC FUNCTION OF PTEROYLGLUTAMIC ACID AND ITS HEXAGLUTAMYL CONJUGATE

I. Hematologic and Urinary Excretion Studies on Patients With Macrocytic Anemia

Frank H. Bethell, M.D., Muriel C. Meyers, M.D., Gould A. Andrews, M.D., Ann Arbor, Mich., and Marian E. Swendseid, Ph.D., Orson D. Bird, Ph.D., and Raymond A. Brown, Ph.D., Detroit, Mich.

THE properties of folic acid as a hematopoietic factor differentiate it clearly from both the dietary extrinsic factor and the antipernicious anemia principle contained in refined liver extracts.[1] Since folic acid occurs in foods almost wholly in conjugated form,[2] it is pertinent to compare the hematopoietic responses of suitable patients with macrocytic anemia following the administration, in sequence, of the vitamin in the bound and free states. Furthermore, an estimate may be obtained of the ability of such patients to release the vitamin from its conjugate by determinations of the urinary excretion of the free material after the oral administration of the conjugate. Because the terminology applied by different authors to folic acid and related compounds is variable and confusing, the free vitamin, folic acid, henceforth will be designated by its chemical name, pteroylglutamic acid, and the conjugate used in these studies will be referred to as pteroylhexaglutamylglutamic acid (hexaglutamyl conjugate).[3] Another conjugate of pteroylglutamic acid has been isolated by Stokstad and co-workers[4] and termed the fermentation *Lactobacillus casei* factor or pteroyltriglutamic acid.

In a previous communication[5] we reported that a naturally occurring conjugated form of folic acid was not as effective as the free vitamin in producing hematopoietic responses in two patients with pernicious anemia in relapse and in one patient with macrocytic anemia following subtotal gastrectomy. Observations leading to a similar conclusion have been described by Welch and associates.[6] At this time we shall present additional data pertaining to the utilization by patients with pernicious anemia and related macrocytic anemias of the hexaglutamyl conjugate as evidenced by their hematopoietic responses and the urinary excretion of the free vitamin after administration of the conjugate. These studies were initiated by the consideration that in pernicious anemia there is an apparent deficiency of the vitamin, pteroylglutamic acid, which cannot, in most cases, be explained by dietary inadequacy.

The specific disturbances of erythropoiesis (namely, megaloblastic proliferation and defective maturation) which characterize many of the macro-

From the Thomas Henry Simpson Memorial Institute for Medical Research, University of Michigan, Ann Arbor, Mich., and the Research Laboratories, Parke, Davis & Company, Detroit, Mich.

Presented in part at the annual meeting of the Central Society for Clinical Research, Chicago, Ill., Nov. 2, 1946.

Received for publication, Dec. 16, 1946.

cytic anemias have suggested that a common functional derangement was operative in their production. This concept was greatly strengthened by the work of Castle and associates[7] who demonstrated convincingly the essential role played by diet and by gastric secretion in the maintenance of normal human erythrocyte formation; it received additional support from the observation that a single form of therapy was generally effective in macrocytic anemias of widely varying clinical background. As a consequence, most studies bearing on the etiology of clinically encountered or experimentally produced macrocytic anemia were concerned with attempts to relate the condition under observation to deficiency of a gastric (intrinsic) or a dietary (extrinsic) factor or to some disturbance in the absorption or utilization of the product of their interaction.[8] Although this theory appeared to be generally consistent with factual observations, its universal application was made difficult by the accumulation of evidence that some cases of macrocytic anemia, particularly those associated with nutritional deficiencies and pregnancy, failed to respond adequately to parenteral administration of concentrated liver principle but showed reversion to normal erythropoiesis when cruder preparations of liver were given either by oral or parenteral routes or when autolyzed yeast was administered by mouth. The existence of a hematopoietic substance in crude liver extracts and yeast, other than the antipernicious factor present in purified liver extracts, was first postulated by Wills and Evans.[9] They concluded from controlled observations that lack of this substance was responsible for their cases of tropical macrocytic anemia as well as for an experimentally produced macrocytic anemia of monkeys[10] and suggested that a similar deficiency in sprue might result from failure of absorption. They identified the substance tentatively as a part of the vitamin B complex and pointed out that it differed from dietary extrinsic factor if the hematopoietic activity of the latter, in accordance with the theory of Strauss and Castle,[8] was limited to a role in the formation of the liver principle.

Day and associates[11] recognized independently in monkeys a macrocytic anemia prevented or cured by administration of yeast or liver extracts. They named the curative factor in these materials vitamin M.[12] More recently, Day and co-workers[13] and also Wilson and associates[14] showed that the deficiency syndrome in monkeys could be successfully treated with purified *L. casei* factor (pteroylglutamic acid). Hogan and Parrott[15] were the first to describe an anemia resulting from a dietary deficiency in chicks. They designated the hematopoietic agent vitamin B_c. Vitamin B_c was subsequently isolated[16] and now has been shown to be identical with pteroylglutamic acid.[3] A similar nutritional deficiency characterized by leucopenia and anemia has been produced in rats largely as a result of the work of Daft, Sebrell, and their associates.[17]

An analysis of the literature dealing with the relative effectiveness of refined liver extracts and cruder preparations of liver, whole liver, or autolyzed yeast in the treatment of human macrocytic anemias was recently published by Watson and Castle.[18] These workers reported four cases of nutri-

tional macrocytic anemia. In three of the patients there was no response to parenterally administered liver extract in dosages effective in the treatment of addisonian pernicious anemia, but there were good hematologic and clinical results when a liver extract was given by mouth. In a fourth patient a response was obtained only when the parenterally administered dose of liver extract was increased ten times. They recognized the presence in crude liver extracts and autolyzed yeast of the hematopoietic substance, described by Wills and Evans,[9] and suggested for it the designation of "Wills' factor."

The observations of Wintrobe[19] on the effects of brewers' yeast on erythropoiesis in pernicious anemia support the view that patients with this disease may be able to utilize some substance other than the "liver principle." Earlier investigators, to whom Wintrobe refers, demonstrated the effectiveness of yeast, usually given in autolyzed form in the treatment of some cases of pernicious anemia, but Wintrobe first seriously questioned the validity of attributing the hematopoietic effect of yeast exclusively to its extrinsic factor content. His data reveal the variability of responses by patients with pernicious anemia to the administration of yeast.

With the demonstration by several groups of investigators of the effectiveness of pteroylglutamic acid in the treatment of pernicious anemia and other macrocytic anemias,[20-24] it appeared probable that the activity of crude liver and yeast in the studies referred to previously was due to their content of pteroylglutamic acid in free or conjugated form.

Earlier observations on the therapeutic effectiveness of pteroylglutamic acid in human anemic and leucopenic states were handicapped by lack of availability of the vitamin in pure form or in relatively concentrated amounts. Thus, the inconclusive or negative results obtained by Sharp and associates,[25, 26] Castle and co-workers,[27] Vilter and Spies,[28] and Moore and associates[22] may be attributed to administration of too small amounts of effective material, as suggested by Sharp, or to the use of material in a form not readily utilizable by persons with pernicious anemia.

CLINICAL MATERIAL AND EXPERIMENTAL PROCEDURE

In the course of this study data were secured from nine patients with addisonian pernicious anemia in relapse and four with other macrocytic (megaloblastic) anemias; of these, two followed gastrectomy, one was probably on a nutritional basis with some features of the nontropical sprue syndrome, and one was associated with chronic diffuse liver disease. Data were also obtained on the urinary excretion of pteroylglutamic acid by three patients with pernicious anemia in liver extract-induced remission. All patients with pernicious anemia fulfilled the diagnostic requirements of macrocytic anemia with characteristic morphologic changes in the peripheral blood, megaloblastic marrow hyperplasia, achlorhydria persisting after parenteral administration of histamine, and ultimate hematopoietic response to treatment with either pteroylglutamic acid or the intramuscular injection of refined liver extract.

Hemoglobin and hematocrit determinations and cell counts were made at regular intervals, using oxalated venous blood, and reticulocyte percentages were obtained daily throughout the period of observation on all patients in relapse. The urinary excretion of pteroylglutamic acid was determined by microbiologic assay using *L. casei*.[2] No evidence was obtained of the excretion of the conjugated vitamin as such. While studies were being carried out, the subjects were given a low protein, meat-free diet of adequate caloric value.

The procedure employed in performing the clinical studies, with some individual modifications, was as follows. Urine collected and suitably preserved during the first twenty-four hours of study was used for the quantitative determination of urobilinogen.[29] Subsequently, for at least two days, the urinary output of pteroylglutamic acid was measured while the patient received the standard diet without medication. A yeast concentrate supplying known amounts of hexaglutamyl conjugate was then given daily for varying periods up to fourteen days, followed by the administration of synthetic pteroylglutamic acid[30, 31] in a dosage equivalent to that of the vitamin present in the conjugate. In one case a period of conjugate administration was followed by one in which the same yeast concentrate was given after incubation with a specific enzyme capable of liberating the vitamin from its bound form. The properties of the hexaglutamyl conjugate are described in the accompanying communication in which are reported the results of urinary excretion studies on normal individuals.[32]

CASE REPORTS AND EXPERIMENTAL RESULTS

I. Hematopoietic Responses of Patients With Macrocytic Anemia Following the Administration of Conjugated and Free Pteroylglutamic Acid

CASE 1.—H. W., a 63-year-old white man, fulfilled all diagnostic criteria of pernicious anemia and had had the disease for approximately four years. Although the anemia was severe the patient appeared to be in fairly good nutritional state, was ambulatory, and had no complicating conditions. He complained of numbness and tingling of the extremities but had no disturbance of gait and no objective signs of central nervous system degeneration. During the preliminary period of observation and diet standardization it became advisable to give the patient a transfusion because of extreme weakness and progression of anemia. Accordingly, 500 c.c. of whole blood were administered and subsequently he was given daily a yeast concentrate containing the equivalent of 4 mg. of pteroylglutamic acid as hexaglutamyl conjugate. After he had received this material for ten days with no hematopoietic response, treatment was changed to synthetic pteroylglutamic acid in a daily oral dose of 4 milligrams. The hematologic data obtained on this patient are shown in Table I.

CASE 2.—N. J., a 56-year-old white man, was first admitted to the Simpson Memorial Institute in 1936, when the diagnosis of pernicious anemia was made and he responded in the expected manner to the parenteral administration of liver extract. During the intervening ten years the patient had received liver therapy very irregularly and none had been taken for several months prior to the present admission. The diet appeared to be adequate particularly with respect to protein from animal sources. There were no subjective or objective manifestations of nervous system involvement. The patient was not given a transfusion, and after a preliminary period without treatment he was given the hexaglutamyl conjugate in a dosage equivalent to 4 mg. of the free vitamin. A small increase in reticulocytes occurred which, since this patient at the same time showed a

TABLE I. HEMATOLOGIC DATA ON CASE 1, H. W.

DATE	TREATMENT	DAY	R.B.C. $\times 10^6$	HEMOGLOBIN (GM.)	HEMATOCRIT (%)	RETICULOCYTES (%)	W.B.C.
2/28/46	None	1	1.1	4.9	13.5	0.6	3,250
3/ 1/46	None	2	--	--	---	0.2	----
3/ 2/46	None	3	--	--	---	0.6	----
3/ 3/46	None	4	--	--	---	0.7	----
3/ 4/46	None	5	--	--	---	0.1	----
3/ 5/46	Blood, 500 c.c.	6	1.0	3.6	12.0	0.2	1,700
3/ 6/46		7 (0)	1.3	4.8	15.5	0.4	1,700
3/ 7/46		1	--	--	---	0.2	----
3/ 8/46		2	--	--	---	0.1	----
3/ 9/46		3	--	--	---	0.1	----
3/10/46		4	--	--	---	0.1	----
3/11/46	Conjugate equivalent to 4 mg. PGA	5	--	--	---	0.1	----
3/12/46		6	--	--	---	0.1	----
3/13/46		7	1.2	4.5	14.0	0.6	----
3/14/46		8	--	--	---	0.7	----
3/15/46		9	--	--	---	0.8	----
3/16/46		10 (0)	1.2	4.2	13.5	0.1	2,960
3/17/46	PGA, 4 mg.	1	--	--	---	0.3	----
3/18/46	PGA, 4 mg.	2	--	--	---	0.6	----
3/19/46	PGA, 4 mg.	3	--	--	---	0.2	----
3/20/46	PGA, 4 mg.	4	--	--	---	0.5	----
3/21/46	PGA, 4 mg.	5	--	--	---	1.4	----
3/22/46	PGA, 4 mg.	6	--	--	---	2.1	----
3/23/46	PGA, 4 mg.	7	--	--	---	2.8	----
3/24/46	PGA, 4 mg.	8	--	--	---	3.2	----
3/25/46	PGA, 4 mg.	9	--	--	---	7.1	----
3/26/46	PGA, 4 mg.	10	1.3	4.2	16.0	9.3	3,050
3/27/46	PGA, 4 mg.	11	--	--	---	7.1	----
3/28/46	PGA, 4 mg.	12	--	--	---	8.2	----
3/29/46	PGA, 4 mg.	13	--	--	---	5.3	----
3/30/46	PGA, 4 mg.	14	1.6	5.0	19.0	4.6	4,600

TABLE II. HEMATOLOGIC DATA ON CASE 2, N. J.

DATE	TREATMENT	DAY	R.B.C. $\times 10^6$	HEMOGLOBIN (GM.)	HEMATOCRIT (%)	RETICULOCYTES (%)	W.B.C.
3/22/46	None	1	2.0	7.9	24.0	1.0	5,500
3/23/46	None	2	--	--	---	0.6	----
3/24/46	None	3	--	--	---	0.7	----
3/25/46		4 (0)	1.8	7.3	23.0	0.8	3,850
3/26/46		1	--	--	---	0.6	----
3/27/46		2	--	--	---	0.6	----
3/28/46		3	--	--	---	1.4	----
3/29/46		4	--	--	---	0.6	----
3/30/46	Conjugate equivalent to 4 mg. PGA	5	--	--	---	2.0	----
3/31/46		6	--	--	---	1.7	----
4/ 1/46		7	--	--	---	3.4	----
4/ 2/46		8	--	--	---	1.4	----
4/ 3/46		9	--	--	---	1.4	----
4/ 4/46		10	1.9	7.4	24.0	4.1	3,250
4/ 5/46		11	--	--	---	3.7	----
4/ 6/46		12 (0)	1.9	7.3	24.0	3.7	3,300
4/ 7/46		1	--	--	---	3.6	----
4/ 8/46	Conjugate equivalent to 4 mg. PGA incubated with conjugase	2	--	--	---	4.0	----
4/ 9/46		3	--	--	---	2.6	----
4/10/46		4	--	--	---	4.1	----
4/11/46		5	--	--	---	2.7	----
4/12/46		6	--	--	---	4.9	----
4/13/46		7	--	--	---	6.9	----
4/14/46		8	--	--	---	4.2	----
4/15/46		9	2.8	9.2	32.5	4.8	7,400

slight rise in urinary excretion level of pteroylglutamic acid, probably indicates some ability to liberate and utilize the conjugated form of the vitamin. During the second part of the experiment the patient received the same yeast concentrate in identical dosage but previously incubated with a specific conjugase derived from hog kidney. This enzyme has been shown to liberate pteroylglutamic acid from the hexaglutamyl conjugate.[2] The quantity of hog kidney tissue employed and the method of preparation of the conjugase-containing extract were such as to render it extremely unlikely that any response could be attributed to the presence of antipernicious substance in hog kidney. A suboptimal but definite reticulocyte rise occurred during administration of this material, together with a substantial increase in erythrocytes and hemoglobin and decided clinical improvement. Hematologic data obtained on this patient are presented in Table II.

Case 3.—H. P., a 65-year-old white man, underwent subtotal gastrectomy for adenocarcinoma of the stomach three years before the present admission. Approximately seven-eighths of the stomach was removed. There were no evidences of metastases at the time

Table III. Hematologic Data on Case 3, H. P.

DATE	TREATMENT	DAY	R.B.C. $\times 10^6$	HEMOGLOBIN (GM.)	HEMATOCRIT (%)	RETICULOCYTES (%)	W.B.C.
2/21/46	None	1	1.8	8.1	25.5	1.2	4,250
2/22/46	None	2	--	--	---	--	----
2/23/46	None	3	--	--	---	1.4	----
2/24/46	None	4	--	--	---	0.6	----
2/25/46	None	5	--	--	---	0.5	----
2/26/46		6 (0)	2.2	7.1	26.5	0.4	4,600
2/27/46		1	--	--	---	0.3	----
2/28/46		2	--	--	---	0.2	----
3/ 1/46	Conjugate	3	--	--	---	0.4	----
3/ 2/46	equivalent to	4	--	--	---	0.6	----
3/ 3/46	2.3 mg. PGA	5	--	--	---	0.4	----
3/ 4/46		6	--	--	---	0.6	----
3/ 5/46		7	--	--	---	1.6	----
3/ 6/46	PGA, 2.3 mg.	8 (0)	1.8	7.5	25.5	1.6	5,400
3/ 7/46	PGA, 2.3 mg.	1	--	--	---	1.8	----
3/ 8/46	PGA, 2.3 mg.	2	--	--	---	1.4	----
3/ 9/46	PGA, 2.3 mg.	3	--	--	---	2.1	----
3/10/46	PGA, 2.3 mg.	4	--	--	---	2.6	----
3/11/46	PGA, 2.3 mg.	5	--	--	---	2.8	----
3/12/46	PGA, 2.3 mg.	6	--	--	---	1.4	----
3/13/46	PGA, 2.3 mg.	7	--	--	---	1.5	----
3/14/46	PGA, 2.3 mg.	8	--	--	---	1.7	----
3/15/46	PGA, 2.3 mg.	9	--	--	---	1.0	----
3/16/46	PGA, 5.0 mg.	10 (0)	2.5	8.5	30.5	1.2	5,800
3/17/46	PGA, 5.0 mg.	1	--	--	---	1.1	----
3/18/46	PGA, 5.0 mg.	2	--	--	---	0.9	----
3/19/46	PGA, 5.0 mg.	3	--	--	---	1.2	----
3/20/46	PGA, 5.0 mg.	4	--	--	---	1.1	----
3/21/46	PGA, 5.0 mg.	5	--	--	---	1.0	----
3/22/46	PGA, 5.0 mg.	6	--	--	---	1.0	----
3/23/46	$FeSO_4$, 2.0 Gm. added	7	2.8	7.5	27.5	0.4	5,950
3/24/46	$FeSO_4$, 2.0 Gm. added	8	--	--	---	0.3	----
3/25/46	$FeSO_4$, 2.0 Gm. added	9	--	--	---	1.2	----
3/26/46	$FeSO_4$, 2.0 Gm. added	10	--	--	---	0.4	----
3/27/46	$FeSO_4$, 2.0 Gm. added	11	--	7.7	29.5	0.6	----
3/28/46	$FeSO_4$, 2.0 Gm. added	12	--	--	---	0.4	----
3/29/46	$FeSO_4$, 2.0 Gm. added	13	3 1	9.6	35.5	0.6	4,000

of the operation or later. Gradual development of weakness and fatigability occurred about one and one-half years before the current admission. Although the dietary habits were modified by the necessity of taking frequent small meals, largely in order to avoid symptoms of postgastrectomy hypoglycemia, the quality of the food was not restricted and, since the patient had received detailed dietary instructions following the operation, the diet may be assumed to have been somewhat better than the average for a person of his age and economic status. The patient's symptoms were attributable to the anemia and physical examination was negative except for pallor and moderate underweight. The urine was negative except for 1 plus protein and the stool examination revealed no occult blood and no evidence of parasitism. This patient received yeast concentrate supplying the hexaglutamyl conjugate in a daily dosage equivalent to 2.3 mg. of the free vitamin for eight days with no hematopoietic response. Subsequently he was given synthetic pteroylglutamic acid in the same dose. There was only a slight increase in reticulocyte percentage but a definite rise in erythrocytes and hemoglobin. Hematologic data are shown in Table III.

CASE 4.—E. B., a 76-year-old white man, fulfilled the diagnostic criteria of pernicious anemia and had had symptoms for approximately two years. The diet had been deficient particularly with respect to meat and vegetables, but the patient had included in his average daily intake six slices of bread, one egg, and one quart of milk. He had lost about 7 pounds during the eight months preceding the admission to the hospital. He had no gastrointestinal complaints other than moderate anorexia. Numbness and tingling of the feet with some difficulty in walking in the dark had been present for three months. The only objective neurologic abnormalities were moderately increased deep reflexes of upper and lower extremities, bilateral, and slight impairment of vibratory sense in the feet. Treatment with yeast concentrate supplying hexaglutamyl conjugate in daily dosage equivalent to 5 mg. of pteroylglutamic acid was carried out for eight days with no hematopoietic response. Since other metabolic studies were performed subsequently on this patient, it was not possible to demonstrate an initial response to administration of the free vitamin alone. However, for three months the patient has received pteroylglutamic acid, 5 mg. daily by mouth, without other medication, and during this period the blood values reached normal levels and remission has been well maintained. Hematologic data obtained on this patient are shown on Table IV.

TABLE IV. HEMATOLOGIC DATA ON CASE 4, E. B.

DATE	TREATMENT	DAY	R.B.C. $\times 10^6$	HEMOGLOBIN (GM.)	HEMATOCRIT (%)	RETICULOCYTES (%)	W.B.C.
8/ 1/46	None	1	1.5	5.8	17.0	2.5	5,100
8/ 2/46	None	2	--	--	---	1.6	----
8/ 3/46	None	3	--	--	---	2.3	----
8/ 4/46	None	4	--	--	---	1.4	----
8/ 5/46	None	5	--	--	---	1.8	----
8/ 6/46	Conjugate equivalent to 5 mg. PGA	6 (0)	1.4	5.3	16.5	2.0	5,200
8/ 7/46		1	--	--	---	2.4	----
8/ 8/46		2	--	--	---	0.8	----
8/ 9/46		3	--	--	---	4.7	----
8/10/46		4	--	--	---	3.1	----
8/11/46		5	--	--	---	3.4	----
8/12/46		6	--	--	---	2.8	----
8/13/46		7	1.4	5.7	16.5	2.8	5,600

CASE 5.—G. L., a 76-year-old white man, complained chiefly of diarrhea and swelling of the legs. Beginning about four years prior to admission to the hospital the diarrhea at first had been periodic, but for the last five months it had been constant with from three to six watery yellow stools each day. Swelling of the legs had been present and had progressed for more than one year. Weakness, ease of fatigue, and dyspnea on exertion had developed over a period of five years, and during this time the patient noted numbness

and tingling of the lower extremities, ataxia, and a burning sensation in his feet. Periodic soreness of the tongue had been present for about one year. He also complained of burning and itching of the eyes. The diet was grossly inadequate, consisting chiefly of bread and potatoes. The patient prepared his own food and bought one-half pound of hamburger each week. No other meat was taken and the diet contained very little milk, eggs, green vegetables, or fruit. His appetite was poor and the patient's weight had decreased from a maximum of 155 pounds to 117 pounds on admission and 100 pounds after diuretic therapy.

Examination revealed the following signs: severe emaciation; dry, shiny skin with many follicular keratoses and irregular areas of brownish pigmentation; vascularizing keratitis and corneal opacities; fissures at the angles of the mouth; a smooth, magenta tongue with some fiery red areas; and other evidences of riboflavin and nicotinic acid deficiency. There was generalized arteriosclerosis. The blood pressure was 108 systolic and 70 diastolic. The heart findings were not abnormal and the lungs were clear except for râles at the bases. The abdomen was negative. Pronounced pitting edema of the lower extremities was present bilaterally. Knee and ankle jerks were not elicited, vibratory sense was absent below the knees, and sense of motion and position was impaired. There was calf muscle tenderness and light pressure over the toes elicited a sensation of burning.

TABLE V. HEMATOLOGIC DATA ON CASE 5, G. L.

DATE	TREATMENT	DAY	R.B.C. $\times 10^6$	HEMOGLOBIN (GM.)	HEMATOCRIT (%)	RETICULOCYTES (%)	W.B.C.
8/19/46	None	1	--	--	---	0.5	----
8/20/46	None	2	--	--	---	1.0	----
8/21/46	None	3	--	--	---	0.3	----
8/22/46		4 (0)	2.4	9.4	29.5	0.5	5,100
8/23/46		1	--	--	---	0.6	----
8/24/46	Conjugate	2	--	--	---	0.7	----
8/25/46	equivalent	3	--	--	---	0.9	----
8/26/46	to 5 mg.	4	--	--	---	1.5	----
8/27/46	PGA	5	--	--	---	1.2	----
8/28/46		6	--	--	---	2.6	----
8/29/46		7	2.8	10.9	34.5	2.9	5,400

Macrocytic anemia of moderate degree was present. Sternal marrow aspiration revealed increased and disordered erythropoiesis with numerous megaloblasts. Histamine refractory achlorhydria and absence of pepsinogen were demonstrated. Total serum proteins were 6.5 Gm. per 100 c.c. with albumin-globulin ratio of 1.6. All other laboratory examinations including hepatic and renal function tests gave essentially normal results. Roentgen study of the gastrointestinal tract revealed profound small bowel dysfunction. "At no point in the course of the small intestine can one find normal bowel pattern. Irregularity of lumen width is pronounced. 'Puddling' of barium is equally obvious." (Dr. F. J. Hodges.)

This patient received the same yeast concentrate as those discussed previously supplying the hexaglutamyl conjugate in a daily dose equivalent to 5 mg. of pteroylglutamic acid. During an eight-day period of conjugate administration little evidence of a reticulocyte response was noted although a significant increase in erythrocytes and hemoglobin occurred (Table V). Moreover, as will be shown later, the patient excreted a relatively large amount of free vitamin during administration of the yeast concentrate. Although this patient obviously suffered with multiple nutritional deficiencies and small bowel disease, it is not possible to exclude addisonian pernicious anemia in view of the achylia gastrica. However, his failure to improve over a period of three months, with persistence of anemia, diarrhea, eye and tongue changes, and further weight loss, strongly suggests that the pathologic process is quite distinct from that responsible for pernicious anemia. Little or no improvement has followed therapy with pteroylglutamic acid in daily doses of

15 mg. by oral and parenteral routes of administration, large doses of refined and crude liver extracts by intramuscular and intravenous injection, and the intravenous administration of plasma and vitamin B fractions. The nervous system manifestations in this case are not severe and may be entirely attributable to peripheral nerve disease. There is no evidence of active infection to explain the failure of response to specific therapy.

CASE 6.—G. W., a 74-year-old white man, developed symptoms of anemia six years prior to admission to the hospital. He was treated with injections of liver extract and improved, but treatment became increasingly irregular. Numbness and tingling of the feet had been present for eighteen months and periodic burning of the tongue for six months. Weakness gradually became so severe that he was unable to leave his home. Although his appetite had been poor for three months and he had lost about 10 pounds during this period, the diet did not appear to be qualitatively seriously deficient. The patient was fairly well nourished but extremely pale. The tongue was smooth with reddening along the lateral margins. Patellar reflexes were diminished and vibratory sense was absent over the left foot and ankle, elsewhere over the lower extremities it was greatly decreased. In other respects the physical findings were essentially normal. The results of laboratory studies, including achlorhydria and megaloblastic marrow reaction, were characteristic of pernicious anemia. This man received a yeast concentrate differing from that given to the patients previously discussed in that by in vitro test it possessed only one seventh as much conjugase inhibitor activity. It has been shown by Bird and associates[33] that yeast extracts contain a strong inhibitor for pteroylhexaglutamylglutamic acid conjugase and that this inhibitor is gradually removed during fractionation of yeast extract in the prepa-

TABLE VI. HEMATOLOGIC DATA ON CASE 6, G. W.

DATE	TREATMENT	DAY	R.B.C. × 10^6	HEMOGLOBIN (GM.)	HEMATOCRIT (%)	RETICULOCYTES (%)	W.B.C.
10/ 7/46	None	1	1.8	7.1	21.0	0.3	4,950
10/ 8/46	None	2	--	--	---	0.4	----
10/ 9/46	None	3	--	--	---	0.4	----
10/10/46	None	4	--	--	---	0.6	----
10/11/46		5 (0)	1.8	6.5	22.0	0.2	3,100
10/12/46		1	--	--	---	0.3	----
10/13/46		2	--	--	---	0.2	----
10/14/46		3	--	--	---	0.4	----
10/15/46		4	--	--	---	0.1	----
10/16/46		5	--	--	---	0.2	----
10/17/46		6	--	--	---	0.5	----
10/18/46	Conjugate, with low conjugase inhibiting activity, equivalent to 4 mg. PGA	7	1.7	6.4	21.0	1.4	4,350
10/19/46		8	--	--	---	2.5	----
10/20/46		9	--	--	---	3.1	----
10/21/46		10	--	--	---	9.1	----
10/22/46		11	1.9	7.3	24.0	11.6	3,550
10/23/46		12	--	--	---	16.2	----
10/24/46		13	--	--	---	9.9	----
10/25/46		14	2.3	8.2	28.0	7.9	6,250
10/26/46		15	--	--	---	3.3	----
10/27/46		16	--	--	---	2.2	----
10/28/46		17	--	--	---	3.5	----
10/29/46		18	--	--	---	2.8	----
10/30/46		19	--	--	---	1.6	----
10/31/46		20	--	--	---	2.8	----
11/ 1/46	PGA, 4 mg.	21 (0)	2.4	8.3	29.0	3.4	3,950
11/ 2/46	PGA, 4 mg.	1	--	--	---	2.7	----
11/ 3/46	PGA, 4 mg.	2	--	--	---	2.0	----
11/ 4/46	PGA, 4 mg.	3	--	--	---	1.5	----
11/ 5/46	PGA, 4 mg.	4	--	--	---	1.2	----
11/ 6/46	PGA, 4 mg.	5	--	--	---	1.8	----
11/ 7/46	PGA, 4 mg.	6	--	--	---	0.8	----
11/ 8/46	PGA, 4 mg.	7	2.8	9.1	32.5	1.0	3,000

ration of pure conjugate. A hematopoietic response was obtained during administration of this yeast concentrate supplying as conjugate the equivalent of 4 mg. of pteroylglutamic acid daily. No second reticulocyte increase followed the oral administration of the same dose of the free vitamin (Table VI).

CASE 7.—T. K., a 69-year-old white woman, fulfilled all diagnostic criteria of pernicious anemia and had first experienced symptoms of anemia five months previously. Numbness and tingling of the feet had been present for about five years with no ataxia or disturbance of gait. The patient was moderately obese and had no evidences of nutritional deficiency. The tongue was reddened and smooth along the lateral margins. Patellar reflexes were diminished and vibratory sensation was impaired over both lower extremities. This patient denied having received any form of antianemia therapy prior to admission, and she was observed in the hospital for fourteen days before treatment was instituted. During this period no hematopoietic response occurred but on the day treatment was started an increase in reticulocyte percentage was observed. The subsequent course of the reticulocyte rise was of a type that strongly indicated a specific response to the material administered, but the high initial value suggested that the marrow at the time of institution of therapy was in a reactive state. She received the same yeast concentrate as that given to G. W. (Case 6) in daily dosage equivalent to 4 mg. of pteroylglutamic acid, and later suggestive but not conclusive evidence was obtained of a second reticulocyte response to treatment with the free vitamin (Table VII).

TABLE VII. HEMATOLOGIC DATA ON CASE 7, T. K.

DATE	TREATMENT	DAY	R.B.C. $\times 10^6$	HEMOGLOBIN (GM.)	HEMATOCRIT (%)	RETICULOCYTES (%)	W.B.C.
10/11/46	None	1	2.5	8.1	30.0	1.2	5,300
10/12/46	None	2	--	--	---	0.8	----
10/13/46	None	3	--	--	---	1.4	----
10/14/46	None	4	--	--	---	2.4	----
10/15/46	None	5	--	--	---	2.4	----
10/16/46	Conjugate, with low conjugase inhibiting activity, equivalent to 4 mg. PGA	6 (0)	2.5	8.6	26.5	5.0	4,200
10/17/46		1	--	--	---	8.2	----
10/18/46		2	--	--	---	7.3	----
10/19/46		3	--	--	---	5.0	----
10/20/46		4	--	--	---	7.4	----
10/21/46		5	--	--	---	10.5	----
10/22/46		6	--	--	---	13.6	----
10/23/46		7	2.9	10.0	30.5	14.1	5,800
10/24/46		8	--	--	---	20.0	----
10/25/46	PGA, 4 mg.	9 (0)	3.0	10.1	31.5	11.9	6,400
10/26/46	PGA, 4 mg.	1	--	--	---	10.1	----
10/27/46	PGA, 4 mg.	2	--	--	---	8.0	----
10/28/46	PGA, 4 mg.	3	--	--	---	14.6	----
10/29/46	PGA, 4 mg.	4	--	--	---	10.6	----
10/30/46	PGA, 4 mg.	5	3.3	10.9	35.0	10.1	6,700

The data obtained from observations of the hematopoietic responses of Cases 1 to 7 indicate that patients with proved pernicious anemia in relapse are unable to utilize in any appreciable degree the hexaglutamyl conjugate of pteroylglutamic acid when the material is supplied orally together with relatively large amounts of a conjugase inhibiting substance. The responses obtained when a conjugate source containing less enzyme inhibitor was administered suggest that the defective utilization of natural forms of pteroylglutamic acid by patients with pernicious anemia is quantitative and probably varies with the individual patient.

II. Urinary Excretion of Pteroylglutamic Acid During Administration of the Hexaglutamyl Conjugate and the Free Vitamin to Patients With Macrocytic Anemia in Relapse

In this study determinations of the twenty-four-hour urinary excretion of pteroylglutamic acid were performed on the patients previously discussed (Cases 1 to 7) and on six additional patients who could not be tested for hematopoietic responses either because of their relatively high initial erythrocyte values or their unwillingness to submit to protracted courses of therapy. The data secured from the urinary assays on all of the subjects are compiled in Table VIII. Brief abstracts of the six additional cases follow.

CASE 8.—O. B., a 39-year-old white man, had had periodic soreness of the tongue for three years and symptoms of anemia for four months. The clinical and laboratory findings were entirely characteristic of pernicious anemia. The patient had received no previous antianemia therapy. Initial hematologic values were: red cells, 1,500,000 per cubic millimeter; hemoglobin, 6.4 Gm. per 100 c.c.; hematocrit, 19 per cent; mean corpuscular volume, 112 cubic microns; mean corpuscular hemoglobin concentration 33.7 per cent; white cells, 4,750 per cubic millimeter. Clinical and hematologic remission was attained while the patient was receiving pteroylglutamic acid, 5 mg. daily.

CASE 9.—L. P., a 63-year-old white woman, had been treated for pernicious anemia with striking symptomatic improvement five years previously. She had had no treatment for about four years prior to the admission. She presented the characteristic features of pernicious anemia with initial blood values as follows: red cells, 900,000 per cubic millimeter; hemoglobin, 3.6 Gm. per 100 c.c.; hematocrit 11.0 per cent; mean corpuscular volume, 122 cubic microns; mean corpuscular hemoglobin concentration, 32.8 per cent; white cells, 3,050 per cubic millimeter. Because of the severity of the anemia a single blood transfusion was given. The patient has subsequently attained complete clinical and hematologic remission while receiving pteroylglutamic acid, 5 mg. by mouth daily.

CASE 10.—H. B., a 71-year-old white woman, had symptoms of weakness, numbness and tingling of the extremities, and soreness of the tongue of approximately one year's duration. She had macrocytic anemia, megaloblastic marrow reaction, and histamine refractory achlorhydria. An initial hematopoietic response was obtained during oral administration of pteroylglutamic acid, 5 mg. daily, but complete remission was induced by intramuscular injection of refined liver extract, 15 units weekly. The hexaglutamyl conjugate was not given to this patient, and urinary excretion data are limited to observations during the administration of the free vitamin. The initial hematologic values were as follows: red cells, 1,700,000 per cubic millimeter; hemoglobin, 6.4 Gm. per 100 c.c.; hematocrit, 20 per cent; mean corpuscular volume, 118 cubic microns; mean corpuscular hemoglobin concentration, 32 per cent; white cells, 4,800 per cubic millimeter.

CASE 11.—D. R., a 55-year-old white woman, had onset of numbness and tingling and disturbances of gait eight months previously. Soreness of the tongue had been present for several weeks. Examination revealed evidence of moderately advanced posterolateral column degeneration. Quantitative blood cell changes were minimal, but definite variations in red cell morphology were observed including anisocytosis and macrocytosis. Histamine refractory achlorhydria was demonstrated. After excretion studies were completed, decided symptomatic improvement and restoration of blood values to normal followed treatment with refined liver extract by intramuscular injection. The initial hematologic data were as follows: red cells, 4,100,000 per cubic millimeter; hemoglobin, 13.3 Gm. per 100 c.c.; hematocrit, 41 per cent; white cells, 4,850 per cubic millimeter.

TABLE VIII. URINARY EXCRETION OF PTEROYLGLUTAMIC ACID BY PATIENTS WITH

CASE NUMBER	DAYS	1	2	3	4	5
Daily excretion of PGA during pretreatment period	1	0.3 γ	1.8 γ	1.4 γ	0.6 γ	1.7 γ
	2	0.5 γ	2.3 γ	2.0 γ	0.4 γ	1.3 γ
	3				0.6 γ	
	4				0.6 γ	
Conjugate daily dose as PGA equivalent		4.0 mg.	4.0 mg.	2.3 mg.	5.0 mg.	5.0 mg.
Daily excretion of PGA during period of conjugate administration	1	1.5 γ	14.8 γ	2.3 γ	4.3 γ	1,195.0 γ
	2	1.6 γ	13.0 γ	3.1 γ	5.8 γ	437.0 γ
	3				5.7 γ	612.0 γ
	4			2.2 γ	33.5 γ	672.0 γ
	5				68.0 γ	900.0 γ
	6				72.0 γ	830.0 γ
	7					1,340.0 γ
	8			2.8 γ		
Average percentage excretion of dose administered		0.028 %	0.30 %	0.04 %	0.337%	17.1 %
PGA as free vitamin; daily dose		4.0 mg.	4.0 mg.	2.3 mg.	5.0 mg.	5.0 mg.
Daily excretion of PGA during period of administration of free vitamin	1	98.8 γ	723.0 γ	45.0 γ	1,115.0 γ	592.8 γ
	2	233.1 γ	761.0 γ	80.0 γ	974.4 γ	420.0 γ
	3				1,082.4 γ	931.3 γ
	4	261.3 γ			1,246.4 γ	980.0 γ
	5					
	6	784.4 γ				
	7					
	8	1,130.0 γ				
Average percentage excretion of dose administered		12.5 %	18.5 %	5.4 %	22.8 %	14.5 %

CASE 12.—T. N., a 56-year-old white man, developed macrocytic anemia five years after total gastrectomy for gastric ulcer. The blood cell values rapidly became normal while the patient was receiving pteroylglutamic acid, 5 mg. daily by mouth.

CASE 13.—E. J. B., a 64-year-old white woman, had macrocytic anemia of mild degree associated with evidences of chronic diffuse liver disease. For one year she had received refined liver extract, 15 units intramuscularly every one to two weeks, and some improvement in blood values had followed this therapy. Shortly before admission to the hospital the patient had an exacerbation of the hepatic disorder with rapidly developing painless jaundice and enlargement of the liver, associated with evidences of increased hepatic dysfunction obtained by laboratory tests. It was during this period of heightened activity of the disease process that pteroylglutamic acid excretion studies were performed. Subsequently the patient recovered from the acute exacerbation of the illness and at present is much improved.

The excretion of free vitamin during oral administration of the hexaglutamyl conjugate to patients with pernicious anemia in relapse and macrocytic anemia following gastrectomy was quite variable. Nevertheless, as has been shown by the percentage calculations on Table VIII, only a small fraction of the dose was excreted in the urine, whereas a much greater part of the orally administered free vitamin was excreted. Comparison of the data obtained on these subjects with those secured during similar studies on normal persons reveals that the patients with pernicious anemia and postgastrectomy macrocytic anemia have much lower excretion levels of pteroylglutamic acid during conjugate administration.[32] Although a difference in excretion values during administration of the free vitamin to these patients and to the normal subjects was also observed, it was much less than that found when the conjugate was given. Moreover, as

MACROCYTIC ANEMIA DURING ADMINISTRATION OF CONJUGATED AND FREE VITAMIN

6	7	8	9	10	11	12	13
0.6 γ	1.3 γ	2.8 γ	0.4 γ	0.4 γ	1.8 γ	2.1 γ	1.2 γ
1.5 γ	0.6 γ	0.9 γ		0.3 γ		2.2 γ	
	0.6 γ	0.9 γ					
	1.6 γ						
4.0 mg.	4.0 mg.	5.0 mg.	4.0 mg.	----	----	4.0 mg.	4.0 mg.
62.0 γ	1.9 γ	3.2 γ	2.0 γ			4.5 γ	2.4 γ
32.0 γ	1.6 γ	2.8 γ	1.4 γ			4.2 γ	2.4 γ
85.0 γ	4.1 γ	5.5 γ					
85.2 γ	8.6 γ		2.3 γ				
72.8 γ	8.6 γ						
44.1 γ	14.9 γ		2.0 γ				
220.0 γ	15.2 γ						
303.6 γ	16.8 γ		2.9 γ				
2.83 %	0.20 %	0.074 %	0.052 %	----	----	0.055%	0.03 %
4.0 mg.	4.0 mg.	5.0 mg.	----	5.0 mg.	5.0 mg.	4.0 mg.	4.0 mg.
339.0 γ	135.2 γ	576.0 γ		219.6 γ	242.6 γ	486.4 γ	9.0 γ
336.0 γ	196.8 γ	1,277.0 γ		499.2 γ	501.7 γ		
	234.3 γ						
	334.6 γ						
	215.0 γ						
				1,876.0 γ			
8.41 %	4.22 %	18.5 %	----	17.3 %	7.4 %	12.13 %	0.2 %

illustrated by Cases 1 and 10, continuous administration of the free vitamin was associated with excretion values increasing to levels equaling those obtained on normal subjects. This was not observed when the hexaglutamyl conjugate was fed continuously in a yeast concentrate possessing strong conjugase inhibiting activity (Cases 3 and 9). Case 4 (E. B.) who also received the conjugate showed a definite increase in excretion of the free vitamin, but the maximum level attained after six days of administration was less than the lowest value observed in the group of normal subjects.

Special consideration must be given to the excretion values obtained in Cases 5, 6 and 7. They will be reviewed together with the pertinent hematologic data in the discussion to follow.

In Case 13, macrocytic anemia associated with liver disease, very low excretion values for pteroylglutamic acid were observed after both conjugate and free vitamin administration.

III. The Effect of Liver Extract (Parenteral) on the Urinary Excretion of Pteroylglutamic Acid During Administration of Its Hexaglutamyl Conjugate to Pernicious Anemia Patients

Refined liver extract was given by intramuscular injection to a patient with pernicious anemia in relapse who had received no previous antianemia therapy (Case 8). The dose employed was 15 U. S. P. units contained in 1 c.c. given on each of three consecutive days. Hexaglutamyl conjugate was taken orally for three days before institution of liver extract therapy and during the period of its administration. No significant change in the excretion levels of pteroyl-

glutamic acid was observed (Table IX). A similar study was carried out on another patient with pernicious anemia (Case 1) after the administration of synthetic pteroylglutamic acid, 10 mg. daily by mouth for seven weeks. During the period of pteroylglutamic acid therapy the erythrocyte count increased from 2,100,000 to 3,300,000 per cubic millimeter. Refined liver extract was injected intramuscularly for six consecutive days in a daily dosage of 15 U. S. P. units except that on the second day of treatment the patient received 45 units. In this case also no change in the excretion of free vitamin was observed during simultaneous administration of hexaglutamyl conjugate and liver extract (Table X).

TABLE IX. EFFECT OF SIMULTANEOUS ADMINISTRATION OF HEXAGLUTAMYL CONJUGATE AND PARENTERAL LIVER EXTRACT ON URINARY EXCRETION OF PTEROYLGLUTAMIC ACID (PGA) BY PATIENT O. B., CASE 8

DATE	TREATMENT	DAY	EXCRETION OF (μG) PGA PER 24 HOURS
9/16/46	None	1	2.3
9/17/46	None	2	0.9
9/18/46	None	3	0.9
9/19/46	None	4	--
9/20/46	Conjugate with high enzyme	1	3.2
9/21/46	inhibitor content equivalent	2	2.8
9/22/46	to 5 mg. PGA	3	5.5
9/23/46		1	5.8
9/24/46	Same plus refined liver extract	2	--
9/25/46	15 units, I. M.	3	5.7

TABLE X. EFFECT OF SIMULTANEOUS ADMINISTRATION OF HEXAGLUTAMYL CONJUGATE AND PARENTERAL LIVER EXTRACT ON URINARY EXCRETION OF PTEROYLGLUTAMIC ACID (PGA) BY PATIENT H. W., CASE 1

DATE	TREATMENT	DAY	EXCRETION OF (μG) PGA PER 24 HOURS
5/28/46	None	1	--
5/29/46	Conjugate with high enzyme	1	33.1
5/30/46	inhibitor content equivalent to 4 mg. PGA	2	
5/31/46	Same plus refined liver extract 15 units, I. M.	1	54.6
6/ 1/46	45 units, I. M.	2	27.2
6/ 2/46	15 units, I. M.	3	14.8
6/ 3/46	15 units, I. M.	4	11.0
6/ 4/46	15 units, I. M.	5	31.9
6/ 5/46	15 units, I. M.	6	16.8

Three patients with pernicious anemia in liver extract-induced remission were studied with respect to their excretion of free vitamin during administration of hexaglutamyl conjugate.

CASE 14.—W. M., a 72-year-old white man, was found to have pernicious anemia thirteen years previously. He has been under continuous observation since then, has received parenteral liver extract regularly, and has never been in relapse.

CASE 15.—N. M., a 64-year-old white woman, was given the diagnosis of pernicious anemia fifteen years before the present studies. Throughout the period she has been treated with parenteral liver extract and the hematopoietic values have been maintained within normal limits.

CASE 16.—A. G., a 66-year-old white man, presented the diagnostic features of pernicious anemia sixteen years previously and for a number of years was treated with desiccated stomach. For the past five years he has received intramuscular injections of liver extract. The patient has always been irregular in following the treatment schedule, and although he has never been in severe relapse, the blood cell values have frequently indicated that therapy was inadequate. When pteroylglutamic acid excretion studies were undertaken the erythrocyte count was 4,100,000 per cubic millimeter, hemoglobin 13.5 Gm. per 100 c.c. and hematocrit 44.5 per cent.

All three of these patients received their initial diagnoses at the Simpson Memorial Institute on the basis of clinical and hematologic features of pernicious anemia, histamine refractory achlorhydria, and subsequent response to antipernicious anemia therapy.

After a preliminary period during which the patients received the standard low protein diet and base line excretion values for pteroylglutamic acid were obtained, they were given the hexaglutamyl conjugate in a daily dose equivalent to 4 mg. of the free vitamin. The source of conjugate was the yeast concentrate possessing strong enzyme inhibiting activity. The last injection of liver extract had been given to these patients about one week previously and none was administered during the excretion studies. The urinary values for pteroylglutamic are presented on Table XI.

It appears to be significant that the excretion levels of pteroylglutamic acid, during the preliminary period when they received the standard diet only, were higher in these patients than in those who were studied during relapse. The base line excretion values of the patients in liver extract-induced remission equal those observed in normal subjects.[32] The excretion of free vitamin during the period of conjugate administration was greater in the patients in remission than in any of the patients with proved pernicious anemia in relapse who received the same yeast concentrate (Table VIII).

TABLE XI. URINARY EXCRETION OF PTEROYLGLUTAMIC ACID (PGA) DURING ORAL ADMINISTRATION OF HEXAGLUTAMYL CONJUGATE AND FREE VITAMIN TO PATIENTS WITH PERNICIOUS ANEMIA IN LIVER EXTRACT-INDUCED REMISSIONS

CASE NUMBER		14	15	16
	DAYS			
Daily excretion of PGA during pretreatment period	1	4.4 μg	2.7 μg	2.5 μg
	2	4.6 μg	1.8 μg	2.2 μg
Conjugate daily dose as PGA equivalent		4.0 mg.	4.0 mg.	4.0 mg.
Daily excretion of PGA during period of administration of conjugate in concentrate with high enzyme inhibitor content	1	147.0 μg	54.5 μg	34.4 μg
	2	268.8 μg	167.0 μg	40.0 μg
	3			71.2 μg
	4			96.6 μg
	5			51.5 μg
	6			173.8 μg
	7			60.8 μg
Average percentage excretion of dose administered		5.1 %	3.0 %	1.8 %
PGA as "free" vitamin; daily dose		4.0 mg.	4.0 mg.	----
Daily excretion of PGA during period of administration of free vitamin	1	564.0 μg	287.5 μg	----
	2	800.0 μg	215.6 μg	
Average percentage excretion of dose administered		16.9 %	6.2 %	

DISCUSSION

It may be presumed that the excretion of pteroylglutamic acid after conjugate administration is dependent upon several factors, aside from renal impairment. These are: (1) the liberation of the vitamin from the conjugate, (2) its absorption from the alimentary tract, (3) the extent of tissue deficiency, and (4) the presence and functional status of tissue enzyme systems required for pteroylglutamic acid utilization.

In pernicious anemia three of these factors may be operative in varying degrees. Liberation of the free vitamin may be adversely affected by defective intestinal conjugase activity aggravated by the presence of enzyme inhibiting substances. The extent of tissue deficiency may influence the retention of absorbed vitamin and so inversely affect its excretion rate. The status of cellular enzyme systems may govern the capacity of the tissues to utilize the vitamin.

The patients in this series who received the hexaglutamyl conjugate together with relatively large amounts of enzyme inhibitor exhibited little or no hematopoietic response. The low excretion levels of pteroylglutamic acid, therefore, may be attributed largely to deficient conversion of the conjugate to the free form. Case 6 (G. W.) who received a conjugate in a preparation with low inhibitor content had moderately high initial and subsequently increasing levels of vitamin excretion. This observation together with the definite but suboptimal hematopoietic response suggests that a significant amount of pteroylglutamic acid was liberated and absorbed but that in spite of tissue need, as shown by the severity of the anemia, there was imperfect utilization of the vitamin. Case 7 (T. K.), at the time of institution of therapy with the same conjugate as that given in Case 6, showed evidences of spontaneous erythrocyte regeneration. The initial reticulocyte percentage was 5.0 and the rise during treatment was rapid, reaching a peak of 20.0 per cent on the eighth day. This value is greater than the expected maximum reticulocyte peak during adequate oral therapy with liver extract or desiccated stomach for a patient with her pretreatment red blood cell count.[34] There can be little doubt that the increase in reticulocytes and total number of circulating red cells, observed in this patient, represents a response to the material administered. The low excretion values for pteroylglutamic acid, as shown on Table VIII, although apparently inconsistent with the observations on other patients in this series, may be attributable to a high degree of tissue deficiency and unusually efficient cellular utilization of the liberated and absorbed vitamin. This concept is supported by the observation in this case of exceptionally low vitamin excretion levels during administration of synthetic pteroylglutamic acid, in the presence of continued hematopoietic response.

Case 5 (G. L.) was discussed earlier with emphasis upon the clinical evidences of multiple nutritional deficiencies refractory to specific therapy. There appear to be adequate grounds for considering this case as one of probable nutritional macrocytic anemia rather than true pernicious anemia. The excretion of unusually high levels of free vitamin during administration of a source of conjugate possessing strong conjugase inhibiting activity is proof of

absorption of the material from the alimentary tract in spite of the small bowel dysfunction and diarrhea. The failure to obtain a sustained hematopoietic response to the subsequent parenteral administration of pteroylglutamic acid and crude and refined liver extracts and the lack of therapeutic effect of large doses of thiamine, riboflavin, and nicotinic acid, administered both orally and parenterally, indicate that in this patient irreversible tissue changes have occurred probably relating to cellular enzyme activity.

These observations suggest that in cases of nutritional macrocytic anemia, even though complicated by extensive disturbances of small bowel function, the liberation and absorption of conjugated pteroylglutamic acid in the presence of conjugase inhibitor are not necessarily impaired as they appear to be in addisonian pernicious anemia.

Some information relative to a possible role of the intrinsic factor may be afforded by the studies on the two patients with macrocytic anemia following gastrectomy (Cases 3 and 12). The patient (Case 3) on whom hematologic data were obtained resembled those with true pernicious failure in the failure to respond to the administration of the hexaglutamyl conjugate. Neither of the gastrectomy patients excreted increased amounts of pteroylglutamic acid when they were given the conjugate. These results indicate that the stomach may be concerned with the liberation of the conjugate and lead to the suggestion that the intrinsic factor, although not itself capable of converting the hexaglutamyl conjugate to the free form,[6] may act enzymatically as an anti-inhibitor.

At this time it is possible only to speculate on the reasons why persons with pernicious anemia in liver extract-induced remission excrete larger amounts of pteroylglutamic acid than those in relapse, either when they are receiving a standard diet or during administration of relatively large amounts of a naturally occurring conjugate of the vitamin. The increased excretion rate observed in remission may be due either to a lessened state of tissue deficiency or to more efficient liberation of the vitamin from its conjugated form. However, both hypotheses may be true, since correction of a tissue deficiency by means of refined liver extract, containing very small quantities of pteroylglutamic acid, implies more effective utilization of natural forms of the vitamin. It should not be assumed, however, that the activity of liver extract in bringing about remission in pernicious anemia is limited to enhancing the availability of dietary sources of pteroylglutamic acid. The observation that pteroylglutamic acid, administered either orally or parenterally, is not equally effective in inducing remissions in all patients with pernicious anemia and that it may have no effect on the combined system degeneration frequently associated with this disease[35] indicates that the action of liver extract is more complex than simply to relieve a conditioned nutritional deficiency of pteroylglutamic acid. The rapidity of therapeutic response to the injection of refined liver extract exhibited by severely ill patients is quite unrelated to their dietary intake and must be attributed to a direct effect upon tissue metabolism. The observations of many investigators of chronic nutritional deficiency states demonstrate that replacement therapy even with excessive amounts of the

deficient nutrilite may be ineffective or only partially effective in re-establishing normal cellular metabolism. Moreover, the protean manifestations of pernicious anemia, and in particular the variable degree of central nervous system involvement found in patients with this disease, suggest that a complex type of cellular enzyme disturbance may exist and that the action of the liver principle in restoring normal pteroylglutamic acid metabolism constitutes but one of its therapeutic effects.

SUMMARY AND CONCLUSIONS

Pteroylglutamic acid (folic acid) and its hexaglutamyl conjugate were given orally to sixteen patients. The series included nine patients with pernicious anemia in relapse, two with macrocytic anemia following gastrectomy, one with chronic liver disease associated with macrocytic anemia, one with macrocytic anemia believed to be due to nutritional deficiency, and three with pernicious anemia in liver extract induced remission.

The hematopoietic responses were observed and the urinary excretion of pteroylglutamic acid was measured during the administration of the conjugated and free vitamin.

Three patients with pernicious anemia in relapse and one patient with macrocytic anemia following gastrectomy exhibited little or no evidence of erythrocyte regeneration when given the hexaglutamyl conjugate in a yeast concentrate containing strong conjugase inhibiting activity. Significant responses occurred when these patients received either equivalent doses of synthetic pteroylglutamic acid or the yeast concentrate after incubation with an enzyme preparation which liberated the free vitamin from its conjugated form.

Two patients with pernicious anemia in relapse had definite hematopoietic responses when they were given the equivalent of 5 mg. daily of pteroylglutamic acid as the hexaglutamyl conjugate in a yeast concentrate containing relatively little enzyme inhibitor.

The patient with probable nutritional macrocytic anemia showed a suboptimal response to the administration of the yeast concentrate with strong conjugase inhibiting activity.

Determinations made prior to the institution of therapy revealed that the urinary excretion of pteroylglutamic acid was lower in patients with macrocytic anemia than in normal subjects or patients with pernicious anemia in liver extract-induced remission.

In patients with pernicious anemia, postgastrectomy macrocytic anemia, and liver disease, the urinary excretion of free vitamin after the oral administration of conjugate with large amounts of inhibitor was much less than the level obtained in normal subjects. One patient with probable nutritional macrocytic anemia excreted the vitamin in amounts comparable to normal subjects.

In one patient with pernicious anemia previously untreated and in another who had been receiving treatment with pteroylglutamic acid without production of complete remission, the simultaneous administration for short periods of refined liver extract by intramuscular injection and the hexaglutamyl con-

jugate by mouth was not followed by increased pteroylglutamic acid excretion above the levels which had been observed when the conjugate was given alone. On the other hand, the administration of conjugated vitamin to three patients with pernicious anemia in liver extract-induced remission was followed by the excretion of the free material in amounts approximately equal to those obtained in normal subjects.

It is concluded that patients with pernicious anemia in relapse and postgastrectomy macrocytic anemia cannot readily utilize for hematopoiesis a naturally occurring conjugate of pteroylglutamic acid although they respond promptly to administration of the free vitamin. This defect is not absolute and varies in different patients. Inability to utilize the conjugated vitamin appears to depend, at least in part, upon a conjugase inhibiting substance present in natural sources of conjugate.

Determination of the urinary excretion of pteroylglutamic acid after conjugate administration provides an index of the ability to liberate the free vitamin from its conjugated form.

One of the pharmacologic actions of the liver principle may be the correction of the metabolic defect in the utilization of naturally occurring conjugated forms of pteroylglutamic acid.

REFERENCES

1. Doan, C. A.: Folic Acid (Synthetic Lactobacillus Casei Factor), an Essential Panhematopoietic Stimulus; Experimental and Clinical Studies, Am. J. M. Sc. **212**: 257, 1946.
2. Bird, O. D., Bressler, B., Brown, R. A., Campbell, C. J., and Emmett, A. D.: The Microbiological Assay of Vitamin B_c Conjugate, J. Biol. Chem. **159**: 631, 1945.
3. Pfiffner, J. J., Calkins, D. G., Bloom, E. S., O'Dell, B. L.: On the Peptide Nature of Vitamin B_c Conjugate From Yeast, J. Am. Chem. Soc. **68**: 1392, 1946.
4. Stokstad, E. L. R., and others: Conference on Folic Acid, New York Academy of Science, May 29, 1946; Nutrition Rev. **4**: 199, 1946.
5. Bethell, F. H., Swendseid, M. E., Bird, O. D., Meyers, M. C., Andrews, G. A., and Brown, R. A.: Observations on the Utilization of Folic Acid (Vitamin B_c) and Vitamin B_c Conjugate by Persons With Pernicious Anemia and Macrocytic Anemia Following Gastrectomy, Univ. Hosp. Bull., Ann Arbor **12**: 42, 1946.
6. Welch, A. D., Heinle, R. W., Nelson, E. M., and Nelson, H. V.: Ineffective Utilization of Conjugated Pteroylglutamic (Folic) Acid in Pernicious Anemia, J. Biol. Chem. **164**: 787, 1946.
7. Castle, W. B.: The Etiology of Pernicious Anemia and Related Macrocytic Anemias, Ann. Int. Med. **7**: 2, 1933.
8. Strauss, M. B., and Castle, W. B.: The Nature of the Extrinsic Factor of the Deficiency State in Pernicious Anemia and in Related Macrocytic Anemias. Activation of Yeast Derivatives With Normal Human Gastric Juice, New England J. Med. **207**: 55, 1932.
9. Wills, L., and Evans, B. D. F.: Tropical Macrocytic Anaemia; Its Relation to Pernicious Anaemia, Lancet **2**: 416, 1938.
10. Wills, L., and Stewart, A.: Experimental Anaemia in Monkeys, With Special Reference to Macrocytic Nutritional Anaemia, Brit. J. Exper. Path. **16**: 444, 1935.
11. Day, P. L., Langston, W. C., and Shukers, C. F.: Leukopenia and Anemia in the Monkey Resulting From Vitamin Deficiency, J. Nutrition **9**: 637, 1935.
12. Day, P. L., Langston, W. C., and Darby, W. J.: Failure of Nicotinic Acid to Prevent Nutritional Cytopenia in the Monkey, Proc. Soc. Exper. Biol. & Med. **38**: 860, 1938.
13. Day, P. L., Mims, V., Totter, J. R., Stokstad, E. L. R., Hutchings, B. L., and Sloan, N. H.: The Successful Treatment of Vitamin M Deficiency in the Monkey With Highly Purified Lactobacillus Casei Factor, J. Biol. Chem. **157**: 423, 1945.
14. Wilson, H. E., Saslaw, S., and Doan, C. A.: The Effect of Folic Acid (Lactobacillus Casei Factor) in Nutritional Hematopenia of Monkeys, J. Lab. & Clin. Med. **31**: 631, 1946.
15. Hogan, A. G., and Parrott, E. M.: Anemia in Chicks Caused by a Vitamin Deficiency, J. Biol. Chem. **132**: 507, 1940.

16. Pfiffner, J. J., Binkey, S. B., Bloom, E. S., Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G., and O'Dell, B. L.: Isolation of Antianemia Factor (Vitamin B_c) in Crystalline Form From Liver, Science **97:** 404, 1943.
17. Daft, F. S., and Sebrell, W. H.: Sulfonamides and Vitamin Deficiencies, Vitamins & Hormones **3:** 58-63, 1945.
18. Watson, J., and Castle, W. B.: Nutritional Macrocytic Anemia, Especially in Pregnancy; Response to a Substance in Liver Other Than That Effective in Pernicious Anemia, Am. J. M. Sc. **211:** 513, 1946.
19. Wintrobe, M. M.: Antianemic Effect of Yeast in Pernicious Anemia, Am. J. M. Sc. **197:** 286, 1939.
20. Spies, T. D.: Folic Acid for Macrocytic Anemia in Relapse, J. A. M. A. **130:** 474, 1946.
21. Darby, W. J., and Jones, E.: Treatment of Sprue With Synthetic Lactobacillus Casei Factor (Folic Acid, Vitamin M), Proc. Soc. Exper. Biol. & Med. **60:** 259, 1945.
22. Moore, C. V., Bierbaum, O. S., Welch, A. D., and Wright, L. D.: The Activity of Synthetic Lactobacillus Casei Factor (''Folic Acid'') as an Antipernicious Anemia Substance. I. Observations on Four Patients: Two With Addisonian Pernicious Anemia, One With Nontropical Sprue, and One With Pernicious Anemia of Pregnancy, J. Lab. & Clin. Med. **30:** 1056, 1945.
23. Goldsmith, G. A.: The Effect of Folic Acid on the Blood Picture in Human Macrocytic Anemia, Federation Proc. **5:** 232, 1946.
24. Zuelzer, W. W., and Ogden, F. N.: Megaloblastic Anemia in Infancy, Am. J. Dis. Child. **71:** 211, 1946.
25. Sharp, E. A., Vonder Heide, E. C., and Wolter, J. G.: Preliminary Clinical Observations on the Antianemia Vitamin B_c (Yeast Concentrate), J. A. M. A. **124:** 734, 1944.
26. Sharp, E. A., and Vonder Heide, E. C.: Failure to Induce Remission of Relapsed Pernicious Anemia by Oral Administration of Vitamin B_c Concentrates, Proc. Central Soc. Clin. Research **17:** 41, 1944.
27. Castle, W. B., Ross, J. B., Davidson, C. S., Burchenal, J. H., Fox, H. F., and Ham, T. H.: Extrinsic Factor in Pernicious Anemia; Ineffectiveness of Purified Casein and of Identified Components of the Vitamin B Complex, Science **100:** 81, 1944.
28. Vilter, C. F., and Spies, T. D.: Quoted by Castle and associates.[27]
29. Watson, C. J., Schwartz, S., Sborov, V., and Bertie, E.: Studies of Urobilinogen; a Simple Method for Quantitative Recording of Ehrlich Reaction as Carried Out With Urine and Feces, Am. J. Clin. Path. **14:** 605, 1944.
30. Angier, R. B., and others: Synthesis of a Compound Identical With the Lactobacillus Casei Factor Isolated From Liver, Science **102:** 227, 1945.
31. Angier, R. B., and others: The Structure and Synthesis of the Liver Lactobacillus Casei Factor, Science **103:** 667, 1946.
32. Swendseid, M. E., Bird, O. D., Brown, R. A., and Bethell, F. H.: Metabolic Function of Pteroylglutamic Acid and Its Hexaglutamyl Conjugate. II. Urinary Excretion Studies on Normal Subjects. Effect of a Conjugate Inhibitor, J. Lab. & Clin. Med. **32:** 23, 1947.
33. Bird, O. D., Robbins, M., Vandenbelt, J. M., and Pfiffner, J. J.: Observations on Vitamin B_c Conjugase From Hog Kidney, J. Biol. Chem. **163:** 649, 1946.
34. Bethell, F. H., and Goldhamer, S. M.: Standards for Maximum Reticulocyte Values Following Ventriculin and Intravenous Liver Extract Therapy in Pernicious Anemia, Am. J. M. Sc. **186:** 480, 1933.
35. Vilter, C. F., Vilter, R. W., and Spies, T. D.: The Occurrence of Combined System Disease in Persons With Pernicious Anemia During Treatment With the Lactobacillus Casei Factor (Folic Acid), Proc. Central Soc. Clin. Research **19:** 26, 1946.

METABOLIC FUNCTION OF PTEROYLGLUTAMIC ACID AND ITS HEXAGLUTAMYL CONJUGATE

II. Urinary Excretion Studies on Normal Persons. Effect of a Conjugase Inhibitor

Marian E. Swendseid, Ph.D., Orson D. Bird, Ph.D., Raymond A. Brown, Ph.D., Detroit, Mich., and Frank H. Bethell, M.D., Ann Arbor, Mich.

THE evidence that pteroylglutamic acid by virtue of its role in erythrocytogenesis[1] is an essential factor in human nutritional economy gives emphasis to a study of the metabolic function of this vitamin. Since pteroylglutamic acid occurs in plant and animal tissues in a conjugated form,[2, 3] such a study must necessarily attempt to delineate the physiologic significance of conjugate in relation to free vitamin and the factors governing conjugate cleavage.

The present communication is concerned with the urinary excretion levels of pteroylglutamic acid (PGA) on normal subjects after the administration of the vitamin both in the free form and as yeast concentrates of the hexaglutamyl conjugate. It was found that there are substances present in certain yeast extracts which lower the expected vitamin excretion rate following conjugate administration, results that are interpreted as evidence of the in vivo action of inhibitors on conjugate conversion.

Bird and associates[4] have reported from the results of their in vitro studies on the cleavage of hexaglutamyl conjugate by the enzyme pteroylglutamic acid (PGA) conjugase that there was a substance present in natural sources (that is, yeast extracts) which caused enzyme inhibition.

The conjugate of pteroylglutamic acid used in this investigation was the peptide, pteroylhexaglutamylglutamic acid. This conjugate has been isolated in crystalline form from yeast,[5] and either it or a conjugate of similar microbiologic activity is widely distributed in both plant and animal tissues.[3] For example, we have found[6] that over 90 per cent of pteroylglutamic acid in the leucocytes and their precursors occur as this type of conjugate. The only other known conjugate is pteroyltriglutamic acid or fermentation factor.[7, 8] Spies[9] has reported a single patient with pernicious anemia in relapse who responded to the intramuscular injection of this material. However, there is no evidence on the basis of differential microbiologic assays* that this conjugate has a general natural distribution, and hence it would appear that it may not be of practical nutritional significance.

From the Research Laboratories, Parke, Davis & Company, Detroit, Mich., and the Thomas Henry Simpson Memorial Institute for Medical Research, University of Michigan Medical Schools, Ann Arbor, Mich.

Received for publication, Dec. 18, 1946.

*The hexaglutamyl conjugate when tested microbiologically has a *Lacto-bacillus casei* activity equivalent to *Streptococcus faecalis* activity; after conjugase treatment *L. casei* and *Str. faecalis* activities are also equivalent but are increased. Pteroyltriglutamic acid has an *L. casei* activity higher than its *Str. faecalis* activity; after conjugase treatment *Str. faecalis* activity increases while *L. casei* activity remains the same.

EXPERIMENTAL

Seven normal individuals took part in the study. Most of them received separately two hexaglutamyl conjugate concentrates as well as the free vitamin in daily doses equivalent to 4 mg. of pteroylglutamic acid. Each preparation was administered orally for two consecutive days and the treatment periods were spaced at intervals of one to four weeks.

The two hexaglutamyl conjugate concentrates used in this study were prepared from yeast. One of the concentrates* was a much purer preparation than the other and contained no conjugase inhibitor. The other concentrate was a norite eluate of yeast and contained conjugase inhibitor. The inhibitor activity of this concentrate relative to the amount of pteroylglutamic acid present as determined by in vitro tests[4] was approximately one-third the concentration found in the original yeast. To split the conjugate present in this concentrate required thirty-six times as many PGA conjugase enzyme units as were necessary to free an equivalent amount of vitamin from crystalline hexaglutamyl conjugate.

The pteroylglutamic acid content of the urine was determined by microbiologic assay using *L. casei*. In some instances analyses were made following treatment of the urine with PGA conjugase from hog kidney to determine whether the vitamin was excreted in the conjugate form. The methods used for the assay and treatment with conjugase have been described.[3]

RESULTS

Tables have been set up showing the urinary excretion levels of PGA before and during the daily administration of 4 mg. of pteroylglutamic acid or concentrates of hexaglutamyl conjugate containing the equivalent of 4 mg. of pteroylglutamic acid.

No evidence for the excretion of the vitamin as the hexaglutamyl conjugate was obtained in any of these experiments, including those where the conjugate was administered as such. These results are not included in the tables.

The values for PGA excretion on unsupplemented diets (averages of 2.5 and 2.9 micrograms on consecutive days) are similar to those studies already reported.[10-12] When pteroylglutamic acid was given, about one-third of the 4 mg. dose was excreted in the urine.

When a concentrate of hexaglutamyl conjugate free from conjugase inhibitor was administered, the urinary PGA excretion levels (averages of 1,193 and 1,412 μg on consecutive days, Table II) were comparable to those obtained when an equivalent amount of the vitamin was given in the free form (averages of 1,266 and 1,288 μg on consecutive days, Table I). However, the administration of conjugate concentrates containing conjugase inhibitor resulted in urinary PGA excretion values (averages of 153 and 244 μg on consecutive days, Table II) which were definitely lower than when the free vitamin or conjugate without inhibitor was given.

*Obtained from Dr. J. J. Pfiffner of the Parke, Davis & Company Laboratories.

TABLE I. URINARY PGA EXCRETION IN NORMAL SUBJECTS BEFORE AND DURING ADMINISTRATION OF 4 MG. PTEROYLGLUTAMIC ACID

SUBJECT	BEFORE TREATMENT (μG PGA PER 24 HR.)		DURING TREATMENT (μG PGA PER 24 HR.)	
	DAY I	DAY II	DAY I	DAY II
He	3.1	2.1	1,381	1,364
Ro	2.3	2.1	1,235	1,221
Ov	2.3	2.9	1,112	1,144
Br	--	3.9	1,184	1,371
Fe	2.9	3.4	1,386	1,374
Bi	2.4	4.1	1,298	1,253
Average	2.5	2.9	1,266	1,288

TABLE II. URINARY PGA EXCRETION IN NORMAL SUBJECTS DURING ADMINISTRATION OF HEXAGLUTAMYL CONJUGATE CONCENTRATES CONTAINING EQUIVALENT OF 4 MG. PGA

SUBJECT	DURING TREATMENT WITH HEXAGLUTAMYL CONJUGATE (μG PGA PER 24 HR.)		DURING TREATMENT WITH HEXAGLUTAMYL CONJUGATE CONTAINING INHIBITOR (μG PGA PER 24 HR.)	
	DAY I	DAY II	DAY I	DAY II
Ro	--	--	155	310
Ov	--	--	260	288
He	2,087	2,184	151	351
Br	1,654	1,807	201	238
Fe	639	1,025	52	143
Bi	517	797	53	163
Sw	1,068	1,248	200	218
Average	1,193	1,412	153	244

TABLE III. URINARY PGA EXCRETION IN NORMAL SUBJECTS DURING ADMINISTRATION OF THE VITAMIN OR ITS HEXAGLUTAMYL CONJUGATE WITH A CONJUGASE INHIBITOR SOURCE

SUBJECT	DAILY TREATMENT	URINARY EXCRETION OF PGA (μG PGA PER 24 HR.)	
		DAY I	DAY II
He	Conjugate (equivalent to 4 mg. PGA) + 30 Gm. Difco yeast extract	110	--
Br	Conjugate (equivalent to 4 mg. PGA) + 30 Gm. Difco yeast extract	76	156
Bi	4 mg. PGA + 30 Gm. Difco yeast extract	1,190	1,030
Sw	4 mg. PGA + 30 Gm. Difco yeast extract	1,350	1,460

To study further the effect of conjugase inhibitor, 30 Gm. of Difco yeast extract as a source of inhibitor was added to the purified conjugate concentrate (the equivalent of 4 mg. of PGA). In two subjects, PGA excretion levels (110 μg and an average of 116 μg per twenty-four hours, Table III) were greatly reduced below the values obtained when the conjugate was given alone.

The urinary PGA excretion levels were also determined during the administration of the free vitamin form together with a source of conjugase inhibitor. The values for each of two subjects (average 1,110 and 1,465 μg per twenty-four hours, Table III) were similar to those obtained when the free vitamin alone was administered (Table I).

DISCUSSION

It has been demonstrated that a hexaglutamyl conjugate concentrate containing PGA conjugase inhibitor when administered to normal subjects results in a lower excretion of PGA than when an equivalent amount of a purer conjugate without inhibitors is given. Moreover, when a natural product containing inhibitor is given with this purer conjugate, urinary excretion levels of PGA are greatly reduced. These data are interpreted as evidence that the PGA conjugase inhibitor can act in vivo on conjugase enzyme systems to reduce the cleavage rate of the hexaglutamyl conjugate and hence to decrease subsequent urinary excretion of the free vitamin. PGA conjugase inhibitor does not affect the excretion level of pteroylglutamic acid when the vitamin is administered in the free form.

It is very possible that the inhibition of the conjugase enzyme system by a dietary source material as observed in this study occurs in the gastrointestinal tract and that at least one of the resultant effects is on the rate of absorption of the vitamin. Either this inhibitor or an endogenous product of similar nature may also be active in internal metabolic cycles to hold the greater portion of the body pteroylglutamic acid content as a conjugate.

While factors affecting in vitro enzyme experiments do not necessarily influence the complex enzyme systems in the body, the possibility must be considered that the same substance inhibits PGA conjugases in vivo and hog kidney PGA conjugase in vitro.[4]

The implications of this in vivo action of PGA conjugase inhibitor are far-reaching. The hexaglutamyl conjugate, the type of compound which serves for the most part as the nutritional source of pteroylglutamic acid, occurs naturally in association with conjugase inhibitor (results of in vitro tests). Such substances as liver, yeast, and spinach, which contain relatively large amounts of conjugate, are also high in inhibitor content.[13] Therefore, from a nutritional and metabolic standpoint, studies involving the role of hexaglutamyl conjugate cannot be considered apart from conjugase inhibitor.

Wright and Welch[10] have stated that, in contrast to other members of the vitamin B group, only a small percentage of the folic acid estimated to be contained in the diet is excreted by normal individuals. It is probable that the data reported here may offer an explanation for this seeming anomaly as regards folic acid in that the action of conjugase inhibitor reduces the rate of cleavage of conjugate to the free vitamin, the form in which pteroylglutamic acid is excreted.

It has been reported that in pernicious anemia there is a lessened ability to utilize conjugated as compared with free pteroylglutamic acid for hematopoiesis. These experiments with normal subjects led to the consideration that in patients with pernicious anemia the PGA conjugase inhibitor in the hexaglutamyl conjugate concentrates[14] might be a factor influencing conjugate utilization, and evidence supporting this idea has been presented in the preceding paper.[15]

SUMMARY

The excretion values of pteroylglutamic acid have been determined for normal persons before and during the daily administration of 4 mg. doses of the vitamin in the free and conjugated forms.

It has been found that for the normal subject given hexaglutamyl conjugate, the urinary excretion levels of pteroylglutamic acid are dependent upon the presence of an exogenous source of conjugase inhibitor. Administering hexaglutamyl conjugate without inhibitor results in PGA excretion levels similar to those obtained following treatment with the free vitamin. Simultaneous administration of conjugase inhibitor and hexaglutamyl conjugate results in lower PGA excretion levels.

REFERENCES

1. Berry, L. J., and Spies, T. D.: The Present Status of Folic Acid, Blood **1**: 271, 1946.
2. Binkley, S. B., Bird, O. D., Bloom, E. S., Brown, R. A., Calkins, D. G., Campbell, C. J., Emmett, A. D., and Pfiffner, J. J.: On the Vitamin B_c Conjugate in Yeast, Science **100**: 36, 1944.
3. Bird, O. D., Bressler, B., Brown, R. A., Campbell, C. J., and Emmett, A. D.: The Microbiological Assay of Vitamin B_c Conjugate, J. Biol. Chem. **159**: 631, 1945.
4. Bird, O. D., Robbins, M., Vandenbelt, J. M., and Pfiffner, J. J.: Observations on Vitamin B_c Conjugase From Hog Kidney, J. Biol. Chem. **163**: 649, 1946.
5. Pfiffner, J. J., Calkins, D. G., O'Dell, B. L., Bloom, E. S., Brown, R. A., Campbell, C. J., and Bird, O. D.: Isolation of an Antianemia Factor (Vitamin B_c Conjugate) in Crystalline Form From Yeast, Science **102**: 228, 1945.
6. Unpublished data.
7. Hutchings, B. L., Stokstad, E. L. R., Bohonos, N., and Slobodkin, N. H.: Isolation of a New Lactobacillus Casei Factor, Science **99**: 371, 1944.
8. Lampen, J. O., and Jones, M. J.: The Antagonism of Sulfonamides by Pteroylglutamic Acid and Related Compounds, J. Biol. Chem. **164**: 485, 1946.
9. Spies, T. D.: The Activity of Another Lactobacillus Casei Factor in Macrocytic Anemia, South. M. J. **39**: 634, 1946.
10. Wright, L. D., and Welch, A. D.: The Production of Folic Acid by Rat Liver in Vitro, Science **98**: 179, 1943.
11. Johnson, B. C., Hamilton, T. S., and Mitchell, H. H.: The Excretion of "Folic Acid" Through the Skin and in the Urine of Normal Individuals, J. Biol. Chem. **159**: 425, 1945.
12. Denco, C. W., Grundy, W. E., Porter, J. W., Berryman, G. H., Freidemann, T. E., and Youmans, J. B.: The Excretion of B-Complex Vitamins in the Urine and Feces of Seven Normal Adults, Arch. Biochem. **10**: 33, 1946.
13. Unpublished data.
14. Bethell, F. H., Swendseid, M. E., Bird, O. D., Meyers, M. C., Andrews, G. A., and Brown, R. A.: Observations on the Utilization of Folic Acid (Vitamin B_c) and Vitamin B_c Conjugate by Persons With Pernicious Anemia and Macrocytic Anemia Following Gastrectomy, Univ. Hosp. Bull., Ann Arbor **12**: 42, 1946.
15. Bethell, F. H., Meyers, M. C., Andrews, G. A., Swendseid, M. E., Bird, O. D., and Brown, R. A.: Metabolic Function of Pteroylglutamic Acid and Its Hexaglutamyl Conjugate. I. Hematologic and Urinary Excretion Studies on Patients With Macrocytic Anemia, J. LAB. & CLIN. MED. **32**: 3, 1947.

THE EFFECT OF THE METHYLATED XANTHINES ON THE CLOTTING TIME OF THE BLOOD

N. C. Gilbert, M.D., Frederick Dey, M.D., and Ruth Trump, B.S.
Chicago, Ill.

THE methylated xanthines, in one form or another, have been in use for over half a century. In the last two decades they have been used very extensively. In spite of occasional adverse criticism, there is good reason to think, from accumulated experimental and clinical evidence, that they are of very definite therapeutic value. Any contraindication for their use should be examined carefully and critically.

It has been reported recently[1] that the methylated xanthines in experimental animals increased the prothrombin level of the blood and increased its coagulability. It would be assumed that if any such action resulted from their clinical use, abundant evidence of such an effect would have been previously observed and reported. On the contrary, it has been our impression, at least, that the patients using one or another of the xanthines over a long period of time had a longer life expectancy, not a shorter. Our experience has been that of Fahr,[2] who has had a long and carefully observed experience with these preparations, that heart failure is apparently postponed.

We have accumulated a great deal of data which we will not attempt to present here in detail, but we will choose only enough to show what we consider very clear evidence that the methylated xanthines have no effect in prolonging the clotting time of the blood.

As a measure of the coagulability of the blood, we determined the clotting time, the response of the subject to intravenous heparin, and the prothrombin level. Clotting times were determined by the capillary tube method. The heparin tolerance test has been adequately described and its significance discussed by de Takats.[3] By his technique, an initial clotting time is determined and then 10 mg. of heparin are injected intravenously. Clotting times are determined at ten-minute intervals until the initial level of the blood is again established.

Prothrombin times were determined on the whole plasma and the diluted (12.5 per cent) plasma, using the technique of Quick. In order to establish the normal prothrombin time for our laboratory, control determinations were made on ten normal individuals. By our technique, the normal time for whole plasma was found to vary between 11 and 12.5 seconds, with a mean of 12; while the time for dilute plasma, with two exceptions, was between 32 and 38 seconds, with a mean of 34. In dogs the normal prothrombin time for whole plasma was 6 seconds and for the 12.5 per cent plasma it was 13 to 17 seconds. The results are the average of duplicate or triplicate determinations.

For the study of the immediate effect of the methylated xanthines upon clotting activity, normal individuals were admitted to the hospital, and the

From the Medical Departments of Northwestern University and St. Luke's Hospital.
Received for publication, Oct. 31, 1946.

coagulability of the blood was determined before and after the administration of the drug. The patients were placed upon a routine, general ward diet. They were treated as ambulatory patients and permitted the freedom of the ward. During the first week in the hospital, daily heparin curves were taken and one or more prothrombin determinations were made. The patients then were given one of the xanthines, usually aminophyllin, either orally or parenterally, and the heparin curves and prothrombin determinations were made at various intervals.

To observe the effect of intramuscular injections, two normal patients were hospitalized and kept under control conditions for the course of the observations. Because of variations of clotting time and the course of the heparin curve, control readings were made for six days. Only two of the curves are plotted in each case. The curves for the other days lay between these two.

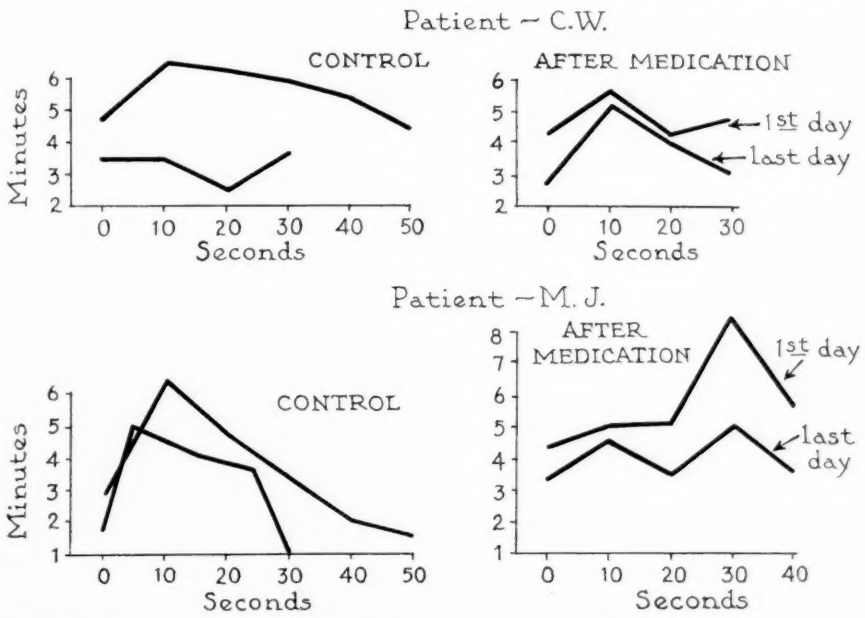


Fig. 1.—Heparin curves before and after the administration of intramuscular aminophyllin, grains 7½, on two patients.

Similarly, after administration of the drug, only the curves of the first and the last day are plotted. These are shown in Fig. 1. In the first patient (C. W.) the control prothrombin readings were 12 seconds for the whole plasma and 33 seconds for the dilute plasma. Prothrombin readings were taken every hour for three hours after the first injection of aminophyllin. These readings were, respectively, 12 seconds for the whole plasma and 34 seconds for the dilute plasma; 12 and 34; 12 and 35; and 12 and 34. At the end of five days the prothrombin times were 12 seconds and 34 seconds.

The second patient (M. J.) showed a control prothrombin time of 12 seconds for the whole plasma and 34 seconds for the dilute plasma. The same readings were obtained at the end of six days.

Two normal patients were given intravenous aminophyllin every eight hours for three doses. The coagulation time and the heparin curve are shown in Fig. 2. The prothrombin times did not change from the controls at the end of the three-day period.

Aminophyllin, 7½ grains intravenously, was also administered to three individuals who were not normal, and the prothrombin levels were determined at hourly intervals for three hours and again at six hours after administration. Two of the patients had been on theobromine calcium salicylate for four weeks; the third was a patient with lymphatic leucemia and had had no medication. The results are shown in Table I.

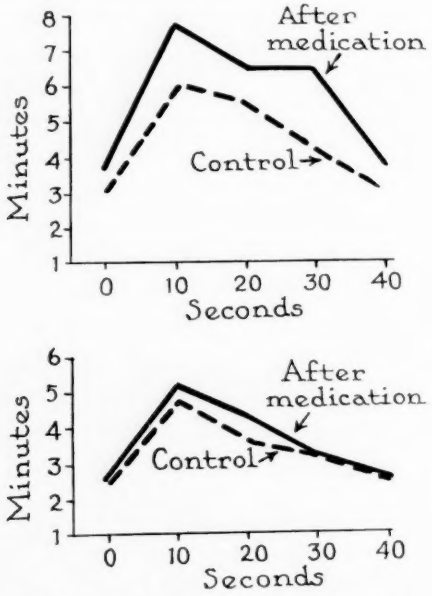


Fig. 2.—Heparin curves before and after the administration of intravenous aminophyllin, grains 7½, on two patients.

Aminophyllin was given to three normal patients in 3 grain doses, three times daily for from fifteen to twenty-eight days. Control prothrombin levels were taken as were heparin curves; prothrombin levels and heparin curves were repeated at intervals of from two days to one week during the administration of the drug. The results were consonant with those obtained in the previous patients and will not be shown in detail. Similar determinations were made on nine patients with cardiac disease who were ambulatory and uncontrolled. These patients took theobromine calcium salicylate, 7½ grains daily by mouth. Normal coagulation times, normal heparin curves, and normal prothrombin levels were present throughout the experiment.

Further experiments were done upon seven dogs. One dog weighing 13 kilograms was given 15 grains of aminophyllin in one dose and was observed for three days. It suffered no ill effects from the large dose. The prothrombin times (in seconds) with the whole and dilute plasma are given in Table II. The coagulation time and heparin curves showed no significant changes from the controls of the previous two days.

TABLE I

	J., AGE 64 (CORONARY OCCLUSION)		B., AGE 42 (CORONARY OCCLUSION)		S., AGE 54 (LYMPHATIC LEUCEMIA)	
	WHOLE PLASMA (SEC.)	DILUTE PLASMA (SEC.)	WHOLE PLASMA (SEC.)	DILUTE PLASMA (SEC.)	WHOLE PLASMA (SEC.)	DILUTE PLASMA (SEC.)
Control	11.5	35	11	34	11	60
1 hour	11	35	12	35	11.5	65
2 hours	12	36	12	35	11	67
3 hours	11.5	38	12	38	11	64
6 hours	11	37	11	38	11	65

TABLE II

	WHOLE PLASMA (SEC.)	DILUTE PLASMA (SEC.)
1 hour	6	13
2 hours	6	14
3 hours	6	13
24 hours	6	14
48 hours	6	15
72 hours	6	14

Another dog weighing 11.3 kilograms was given 135 mg. of aminophyllin three times a day for two weeks. This dose was chosen in order to duplicate an experiment in which changes in prothrombin time were noted in such an experiment elsewhere. The first control reading and the heparin curve and the last heparin curve at the termination of the experiment are given in Fig. 3. The two prothrombin readings before the experiment were 6 and 15 seconds. During the experiment the prothrombin times varied from 6 and 15 seconds to 6 and 17 and were 6 and 16 on the last day. The other experiments upon dogs were repetitions of the clinical experiments and developed nothing different from the results shown. Aminophyllin was used in six experiments and theobromine calcium salicylate in one. The results throughout these experiments upon animals were in complete accord with those of Quick.[5]

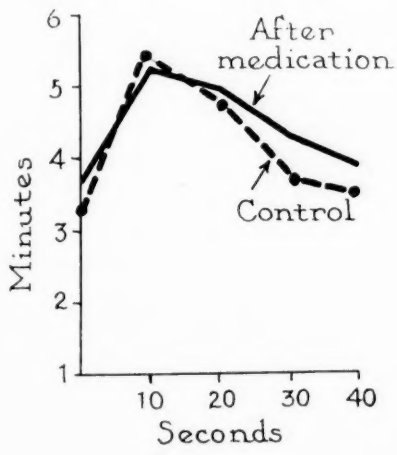


Fig. 3.

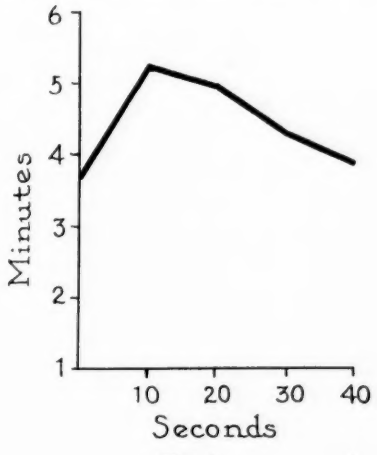


Fig. 4.

Fig. 3.—Dog given 135 mg. of aminophyllin three times a day for two weeks. Control heparin curve and heparin curve at termination of experiment.

Fig. 4.—Composite heparin curve derived from averages on patients who had taken methylxanthines for from ten days to twenty years.

In order to determine whether the prolonged administration of the methylated xanthines has any effect upon clotting activity, studies were made of twenty-five patients chosen at random who had been taking one or another of these preparations for from ten days to twenty years. The results are shown in Table III. Heparin curves were taken in each case. A composite curve is shown; it is based upon the arithmetical averages of each period after the administration of heparin (Fig. 4). The two extremes are shown in Fig. 5. In each case the patient had been taking aminophyllin for two years.

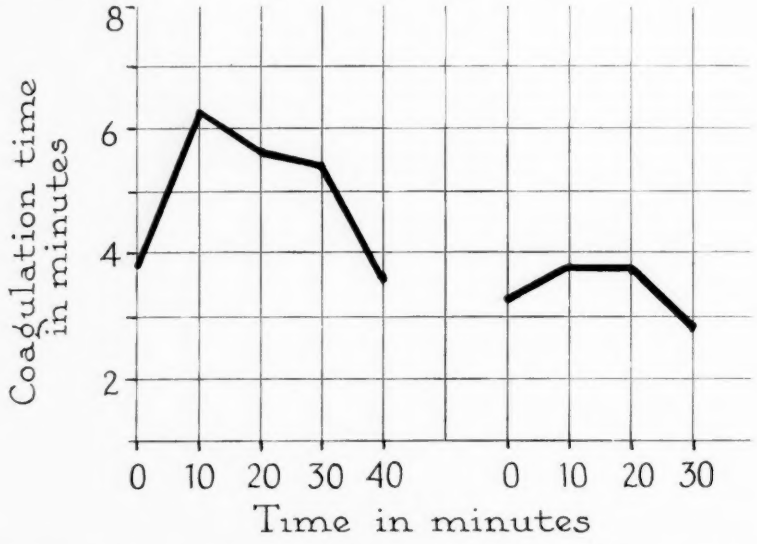


Fig. 5.—These two curves represent the two extremes on patients taking aminophyllin over long periods of time. In each case aminophyllin had been taken continuously for two years.

After the foregoing work was done, Scherf and Schlachman[6] reported a shortening of prothrombin time following the intravenous administration of aminophyllin and theobromine sodium acetate. Rieben,[7] on the other hand, reported that he was unable to confirm the work of Link showing the shortening of the prothrombin time to result from the administration of the methylated xanthines.

We are conscious of the conflicting and confusing reports which have appeared on this subject. A very ready recourse is available for determination of the effects of these drugs by following the advice of Hunter to Jenner—"Don't think. Try!" The determination of the coagulation time by the capillary tube method is easily done, requires no special apparatus, and is accurate for clinical purposes. Allowance must be made for normal, minor deviations from day to day, depending upon several variables. Determinations of prothrombin time admit of many errors. This is especially true when different modifications are used which introduce doubtful variables. It would add to the value of comparative studies if a standard method, such as that of Quick, were used. After all, however, the real point, as far as the methylated xanthines are concerned, is whether the coagulation of the blood is or is not hastened. This can readily be determined in any doctor's office.

TABLE III. CLOTTING ACTIVITY OF PATIENTS RECEIVING METHYLATED XANTHINES FOR LONG PERIODS OF TIME

PATIENT	PERIOD OF MEDICATION	CLOTTING TIME (MIN. AND SEC.)	PROTHROMBIN TIME	
			WHOLE PLASMA (SEC.)	DILUTE PLASMA (SEC.)
1	10 days	3	12	34
2	21 days	4	12	36
3	5 weeks	4	12	36
4	4 months	3, 20		
5	5 months	4, 15	12	36
6	6 months	3, 40	12.5	36
7	18 months	3, 30		
8	2 years	3, 15	12.5	34
9	2 years	3, 45	12	36
10	2.5 years	3, 50	11	33
11	3.5 years	4, 40		
12	3 years	5, 45	12.5	36
13	3 years	3, 00	12	35
14	3.5 years	4, 00	11	33
15	4 years	5	11.5	32
16	5 years	3, 45	12.5	34
17	5.6 years	3, 30		
18	6 years	3	11.5	33
19	6 years	3, 30		
20	7 years	3, 40		
21	8 years	6	12	34
22	10 years	2, 30		
23	14 years	4, 35		
24	16 years	3, 00	12.5	35
25	20 years	4	11.5	33

SUMMARY

A series of experiments were carried out on patients and on animals to determine whether the prothrombin time, the clotting time of the blood, and the heparin curve were in any way changed by the administration of the xanthine drugs. Prothrombin times were determined in the whole plasma and in the dilute plasma. No change was found by either method. Coagulation times were not affected. The heparin curves were either not affected or were improved.

We wish to express our indebtedness to Dr. Armand J. Quick for his kindly help and advice in the methods of determining prothrombin time and in perfecting our technique.

REFERENCES

1. Field, J. B., Larsen, E. G., Spero, L., and Link, P.: Studies on the Hemorrhagic Sweet Clover Disease. XIV. Hyperprothrombinemia Induced by Methylxanthines and Its Effect on the Action of 3,3′-Methylenebis-(4-Hydroxycoumarin), J. Biol. Chem. **156:** 725, 1944.
2. Fahr, G.: Personal communication.
3. de Takats, G.: Heparin Tolerance; a Test of the Clotting Mechanism, Surg., Gynec. & Obst. **77:** 31, 1943.
4. Quick, A. J.: The Hemorrhagic Diseases and the Physiology of Hemostasis, Springfield, Ill., 1942, Charles C Thomas, Publisher, p. 312.
5. Quick, A. J.: Experimentally Induced Changes in the Prothrombin Level of the Blood. Quantitative Studies in Dogs Given Dicumarol. II. The Effect of Methylxanthines on Prothrombin per se and When Administered With Dicumarol, J. Biochem. **161:** 33, 1945.
6. Scherf, D., and Schlachman, M.: The Effect of Methylxanthines on the Prothrombin Time and Coagulation of the Blood, Am. J. M. Sc. **212:** 83, 1946.
7. Rieben, W. K.: Beobachtungen mit einer neuen Prothrombin Bestimmungsmethode, Helvet. med. acta **13:** 295, 1946.

PENICILLIN IN BEESWAX AND PEANUT OIL, A NEW PREPARATION WHICH IS FLUID AT ROOM TEMPERATURE: ABSORPTION AND THERAPEUTIC USE

HAROLD L. HIRSH, M.D., HARRY F. DOWLING, M.D., JEAN J. VIVINO, M.D., AND GEORGINE ROTMAN-KAVKA, M.D.
WASHINGTON, D. C.

PENICILLIN is administered by intermittent intramuscular injections more frequently than by any other method. While continuous intravenous and intramuscular infusions may be best for maintaining high concentrations of the antibiotic over a long period of time, they are necessarily restricted to patients who are closely confined. Likewise, oral administration can be used only in the treatment of certain groups of patients, particularly in those with diseases that respond easily and quickly to penicillin. The major disadvantage of administering penicillin intramuscularly in aqueous solution is the necessity for repeating the injections at two- or three-hour intervals. Consequently, several attempts have been made to slow the absorption of penicillin after it is deposited within the muscles; the most successful of these attempts has been the incorporation of the antibiotic in beeswax and peanut oil by Romansky and Rittman.[1] A single injection of 1 c.c. of their preparation, containing 300,000 units of penicillin, is usually folllowed by therapeutically effective concentrations in the blood for four to twenty-four hours.[2, 3] There are certain technical difficulties, however, in the administration of this mixture when it is made according to the original formula. The material is in a solid state at room temperature, and in order to liquefy must be heated before it is injected.

We have studied a preparation containing pure penicillin G which is a modification of the original formula. Merck & Company, Inc., from whom it was obtained, state that the procedure employed in its manufacture "alters the physical state of the menstruum and substantially reduces the viscosity at all temperatures."[4*] We found that this material became fluid within fifteen to thirty minutes after it was removed from the refrigerator to room temperature and that it was not necessary to heat it before it was administered. In the present paper we are reporting the results of our study of the absorption of this mixture and of its use in patients with various infections.

STUDIES ON ABSORPTION

Both crystalline sodium penicillin G and purified calcium penicillin G were administered to an equal number of patients. We found no difference between the two salts, except that the sodium penicillin was lighter in color and became fluid more quickly at room temperature. Each cubic centimeter contained

From the George Washington University Medical Division, Gallinger Municipal Hospital, and the Department of Medicine, George Washington University School of Medicine.
Received for publication, Nov. 1, 1946.
*Stable at room temperature for at least six months.

300,000 units in refined peanut oil containing 4.8 per cent (W/V) of U. S. P. bleached beeswax. It was administered by using separate 18 or 20 gauge needles for withdrawal and for injection. It was found necessary that both the syringes and the needles be *absolutely* dry.

Twenty subjects without any evidence of impaired renal function or of cardiac or hepatic failure were given 300,000 units of the penicillin, contained in 1 c.c. of wax and oil, deep in the gluteal muscles. Eleven patients received the sodium salt and nine the calcium penicillin, but since no differences were observed in the serum concentrations obtained the two preparations will be considered together. Blood for penicillin determinations was taken at three-hour intervals for twelve hours starting twelve hours after injection in sixteen subjects and during the whole twenty-four hour period following injection in four patients. Serum concentrations of penicillin were determined by a modification of the method of Rammelkamp.[5] The results are shown in Table I. None of the patients had an assayable concentration of penicillin in the serum at twenty-four hours. Five subjects had significant concentrations at twenty hours, ten at eighteen hours, and fourteen at fifteen hours. All but two of the subjects had detectable concentrations of the antibiotic in their serum at twelve hours. Concentrations ranging from 0.625 to 5 units per cubic centimeter of serum were found in four subjects three to nine hours after injection. In two subjects concentrations of 1.25 and 0.156 units per cubic centimeter were found in the serum fifteen and thirty minutes, respectively, after injection.

TABLE I. SERUM CONCENTRATIONS OF PENICILLIN (UNITS PER CUBIC CENTIMETER) FOLLOWING SINGLE INTRAMUSCULAR INJECTIONS OF 300,000 UNITS (1 C.C.) OF PENICILLIN-BEESWAX-PEANUT OIL MIXTURE

PATIENT	3 HR.	6 HR.	9 HR.	12 HR.	15 HR.	18 HR.	21 HR.	24 HR.
9				0	0	0	0	0
15				0	0		0	0
11				1.25	0	0	0	0
12				0.312	0	0	0	0
16				0.039	0		0	0
8				0.156	0.078	0	0	0
10				0.312	0.039	0	0	0
17				0.625	0.078		0	0
18				0.312	0.078		0	0
2	2.5	1.25	1.25	1.25		0	0	0
1	1.25	0.625	0.625	0.625		0.039		0
7				0.625	0.156	0.039	0	0
3	5.0	1.25	1.25	1.25		0.078		0
4	5.0	5.0	5.0	5.0	2.5	0.625	0	0
13				1.25	0.156	0.078	0	0
6					0.625	0.078	0.078	0
5				2.5	0.625	0.625	0.312	0
14				5.0	0.078	0.078	0.078	0
19				0.625	0.312		0.039	0
20				0.625	0.156		0.039	0

Blood was taken on two occasions from one patient who was receiving daily injections of 300,000 units twenty-four hours after a preceding injection, and no penicillin was detectable.

Since significant concentrations of penicillin were found in the serum of most of the subjects studied for only twelve hours following the injection of 300,000 units, it was decided to study the absorption of 600,000 units of penicillin in wax and oil, contained in 2 c.c. of the mixture. Thirty-six subjects

were given either the sodium or the calcium preparation deep in the thigh or gluteal muscles. Blood for penicillin determination was taken at four-hour intervals thereafter for twenty-four hours in four subjects and at the same intervals starting twelve hours after injection in thirty-one patients. The results are shown in Table II. Twelve of the thirty-six subjects studied had assayable concentrations of penicillin in their serum at twenty-four hours, twenty at twenty hours, and twenty-nine at sixteen hours. In only two patients was there no evidence of penicillin in the serum at twelve hours. Concentrations of penicillin ranging from 0.312 to 10 units per cubic centimeter were found in the serum of five subjects four and eight hours after the injections. Two subjects had serum concentrations of 1.25 units per cubic centimeter thirty minutes after administration of the mixture.

Kirby and associates[6] found that a higher percentage of patients had detectable concentrations of penicillin in their blood at twenty-four hours following subcutaneous injection of 600,000 units of penicillin in beeswax and peanut oil than following intramuscular injection of a similar amount. Consequently, we gave twenty subjects 600,000 units of penicillin, contained in 2 c.c.

TABLE II. SERUM CONCENTRATIONS OF PENICILLIN (UNITS PER CUBIC CENTIMETER) FOLLOWING SINGLE INTRAMUSCULAR INJECTIONS OF 600,000 UNITS (2 C.C.) OF PENICILLIN-BEESWAX-PEANUT OIL MIXTURE

PATIENT	4 HR.	8 HR.	12 HR.	16 HR.	20 HR.	24 HR.
3			0	0	0	0
14	2.5	0.625	0	0	0	0
12	10.0	0.312	0.078	0	0	0
9			0.156	0	0	0
8			0.156	0	0	0
31			0.156	0	0	0
32			0.156	0	0	0
15	2.5	1.25	0.078	0.039	0	0
16			2.5	0.625	0	0
18			5.0	0.625	0	0
23			0.312	2.5	0	0
28			0.625	0.156	0	0
29			10.0	0.625	0	0
33			1.25	0.156	0	0
34			0.625	0.156	0	0
35			0.625	0.078	0	0
7			0.156	0.156	0.078	0
6			0.625	0.312	0.078	0
5			0.625	0.312	0.078	0
2			1.25	0.625	0.156	0
20			5.0	1.25	0.156	0
11			2.5	0.625	0.312	0
17			5.0	5.0	0.625	0
36			0.625	0.156	0.156	0
13	10.0	1.25	0.625	0.312	0.078	0.078
10			0.625	0.625	0.312	0.156
21			1.25	0.625	0.156	0.156
1			2.5	1.25	0.312	0.156
4			>40.0	5.0	1.25	0.156
19			1.25	1.25	0.312	0.312
22			2.5	1.25	0.625	0.156
24			2.5	1.25	1.25	1.25
25			1.25	1.25	1.25	1.25
26			10.0	10.0	0.625	0.312
27			20.0	10.0	0.625	0.625
30			10.0	2.5	0.312	0.312

of wax and oil, subcutaneously. Blood was obtained at four-hour intervals for twelve hours starting twelve hours after injection in thirteen subjects and at four, eight, sixteen, twenty-four, and twenty-eight hours after injection in seven subjects. The results are shown in Table III. All but one of the patients had detectable concentrations of penicillin in the serum at twenty-four hours. In two subjects absorption was irregular.

TABLE III. SERUM CONCENTRATIONS OF PENICILLIN (UNITS PER CUBIC CENTIMETER) FOLLOWING SINGLE SUBCUTANEOUS INJECTIONS OF 600,000 UNITS (2 C.C.) OF PENICILLIN-BEESWAX-PEANUT OIL MIXTURE

PATIENT	4 HR.	8 HR.	12 HR.	16 HR.	20 HR.	24 HR.	28 HR.
1			0.156	0.039	0	0	
2	2.5	5.0		0.312		0.078	0
3	10.0	10.0		0.078		0.078	0
4			10.0	0.312	0.039	0.039	
5			0.625	0.312	0.312	0.078	
6			10.0	0.156	0.156	0.156	
7			0.625	0.625	0.156	0.156	
8			0.312	0.312	0.312	0.156	
9			0.312	0.156	0.156	0.156	
10			0.156	0	0.156	0.156	
11			20.0	0.625	1.25	0.312	
12			5.0	2.5	1.25	0.312	
13			2.5	2.5	0.625	0.312	
14			0.625	0.312	0.312	0.312	
15			1.25	0.625	0.625	0.312	
16	40.0	5.0		0.625		0.078	0.078
17	2.5	1.25		0.625		0.625	0.156
18	5.0	2.5		0.625		0.312	0.156
19	2.5	0.625		0.625		0.312	0.312
20	2.5	1.25		0.312		0.125	0.312

CLINICAL STUDY

Thirty-two patients with various infections have been treated with either sodium or calcium penicillin in wax and oil. In Table IV are shown the results of the treatment of these infections and the dosage schedules employed. The seven patients with gonorrhea had previously failed to respond to doses up to 400,000 units of commercial penicillin in aqueous solution and consequently were treated with a single injection of 600,000 units of penicillin in wax and oil. All had clinical and laboratory evidences of prompt and complete cure which were verified by follow-up examinations two, seven, fourteen, and twenty-one days after treatment. Six patients with gonococcal arthritis were treated with doses of 300,000 to 600,000 units every twelve hours. One patient failed to respond to a course consisting first of 300,000 units and later of 600,000 units given twice a day for a total of fourteen days. No improvement was noted despite the fact that penicillin concentrations of 0.625 unit per cubic centimeter were found in the joint fluid, whereas only 0.039 unit per cubic centimeter were required to inhibit the gonococcus in vitro. There was no response to an additional course of penicillin in isotonic salt solution, 50,000 units intramuscularly every three hours for five days. The patient ultimately recovered coincident with the administration of sulfadiazine.

Two patients with salpingo-oophoritis were treated with penicillin in wax and oil. The causative agent in the case of one patient was the gonococcus; in

TABLE IV. RESULTS OF CLINICAL STUDY OF PENICILLIN IN BEESWAX AND PEANUT OIL

DISEASE	NUMBER OF PATIENTS	PLAN OF THERAPY (UNITS)	RESULTS
Gonorrhea	7	600,000	Recovered
Gonococcal arthritis	6	300,000 to 600,000 once or twice a day for 8 to 10 days	5 Recovered 1 Unimproved
Salpingo-oophoritis	2	300,000 twice a day for 5 days	Recovered
Secondary syphilis	1	300,000 once a day for 16 days	Serologic titer zero 3 months after completion of therapy
Subacute bacterial endocarditis	1	300,000 twice a day for 42 days, then 600,000 once a day for 14 days	No evidence of infection 2 months after completion of therapy
Infections of skin	9	300,000 to 600,000 once or twice a day for 3 to 14 days	Recovered
Erysipelas	1	600,000 once a day for 5 days	Recovered
Otitis media	2	600,000 once a day for 5 to 8 days	Recovered
Tonsillitis and pharyngitis	2	600,000 once a day for 5 days	Recovered
Laryngitis and bronchitis	1	600,000 once a day for 5 days	Unimproved

the other the agent was not identified. Both patients noted marked relief of pain and discomfort within twenty-four hours after treatment was started. The temperature returned to normal within this period of time. Convalescence was uneventful and the drug was discontinued after five days.

One patient with secondary syphilis was given a course of penicillin in wax and oil consisting of 300,000 units once a day for sixteen days for a total of 4,800,000 units. The course of therapy was uneventful, and three months after completion of treatment the patient's serologic titer had fallen to zero.

Penicillin in beeswax and peanut oil was administered to one patient with subacute bacterial endocarditis due to a *Streptococcus viridans*. He made a spectacular response following the initiation of treatment with his preparation in doses of 300,000 units every twelve hours. This was later changed to 600,000 units once a day. The patient made an uneventful recovery following fifty-six days of treatment and has remained free of evidences of infection two months after completion of therapy.

Nine patients with cellulitis and one with erysipelas were treated with doses of 300,000 to 600,000 units once or twice a day for three to ten days with complete resolution of the infection in each case. In one patient the area of cellulitis surrounded a large abscess which was incised and drained. A hemolytic *Staphylococcus aureus* was cultured from the pus. Still another patient developed scarlet fever in addition to the cellulitis, and recovery was uneventful despite the complication.

Two patients with acute catarrhal otitis media and one patient with tonsillitis and pharyngitis caused by a hemolytic streptococcus recovered with the use of this preparation of penicillin in doses of 600,000 units once a day for five

to eight days. The only other therapeutic failure was in a patient with a nonspecific laryngitis and bronchitis treated with 600,000 units of penicillin once a day for five days.

DISCUSSION

The injection of 300,000 units of penicillin in wax and oil resulted in concentrations of at least 0.156 unit per cubic centimeter of serum twelve hours after administration in seventeen of twenty patients (85 per cent). An additional patient exhibited a concentration of 0.039 unit per cubic centimeter increasing to 90 per cent the incidence of significant concentrations of the antibiotic in the serum at twelve hours. These concentrations of penicillin have been found to be bactericidal for most penicillin-sensitive organisms. Seventy per cent of the patients studied had serum concentrations of 0.039 unit per cubic centimeter, or more, fifteen hours after the injection; 50 per cent exhibited these concentrations at eighteen hours and 25 per cent at twenty-one hours.

Increasing the dose to 600,000 units resulted in serum concentrations of 0.078 unit per cubic centimeter, or more, at twelve hours in thirty-four out of thirty-six patients (94 per cent). Twenty-nine patients (80 per cent) had effective concentrations of the antibiotic in their serum at sixteen hours and nineteen (54 per cent) at twenty hours. One-third of the patients studied were found to have serum concentrations of penicillin of at least 0.078 unit per cubic centimeter twenty-four hours after injection.

The injection of 600,000 units of penicillin in wax and oil subcutaneously resulted in high concentrations for as long as twenty-four hours after injection in all except one of the patients studied.

The concentrations of penicillin in the blood reported by Kirby and associates[3] following the intramuscular injection of 300,000 units of penicillin in beeswax and peanut oil were present for a shorter time than those reported by Romansky and Rittman[2] who used a similar preparation.

Both of these groups of investigators employed a preparation made according to the original formula devised by Romansky. Our results with the modified formula are essentially the same as those obtained by Kirby and associates.[3]

Thirty-two patients with various infections amenable to penicillin were treated with this preparation. The results were as satisfactory as those obtained with penicillin administered in aqueous solution or with penicillin made according to the original formula.[7-12]

Only slight to moderate local pain occurred when the preparation was injected intramuscularly. This discomfort began immediately and usually lasted only a minute or two, very occasionally as long as three or four hours. Most of the patients complained of moderate to marked pain immediately after subcutaneous injection and developed a painful tender nodule which persisted for several days. Eleven days after a subcutaneous injection of 600,000 units of penicillin, one patient developed an area of induration and angioneurotic edema 3 inches in diameter without constitutional symptoms. This subsided within a week on symptomatic measures.

It is obvious that any modification of penicillin which will permit injections to be spaced at twelve- to twenty-four hour intervals will be advantageous, particularly in the case of patients with such diseases as subacute bacterial endocarditis or syphilis who have to be treated for long periods of time. Therapy is simplified in infections which require treatment for several days. This method of treatment is ideal also for patients with gonorrhea since it can be carried out with one injection. The high serum concentrations obtained during the first few hours following injection make it likely that infections caused by organisms which are relatively resistant to penicillin can be treated satisfactorily by proper regulation of the dose and time interval. These advantages have been offset, in the past, by the difficulty inherent in the administration of penicillin in peanut oil and beeswax as prepared by the original Romansky formula. We believe that the modified preparation which we have used eliminates this difficulty in administration, at the same time retaining all the other advantages of the original preparation.

SUMMARY AND CONCLUSIONS

1. A new preparation of penicillin incorporated in peanut oil and beeswax has been studied to determine its rate of absorption and its therapeutic efficacy.

2. The intramuscular injection of 1 c.c. containing 300,000 units of penicillin resulted in assayable concentrations of penicillin in the serum for twelve hours in 85 per cent of the patients studied, with measurable concentrations persisting for as long as twenty-one hours in 25 per cent. Following an intramuscular injection of 2 c.c. containing 600,000 units, detectable concentrations were found in 90 per cent of the subjects at twelve hours and in one-third of the patients for twenty-four hours. An injection of 600,000 units subcutaneously resulted in detectable concentrations of penicillin in the blood for twenty-four hours in all but one of the subjects studied.

3. Twenty-seven patients suffering from various infections were treated with doses varying from 300,000 to 600,000 units once or twice per day. The results were similar to those obtained with the use of other preparations of penicillin in beeswax and oil or with equivalent *per diem* doses of penicillin in aqueous solution.

4. The preparation used by us was found to be easier to administer than the preparations made according to the original Romansky formula.

We wish to thank Dr. William W. Zeller, for his assistance in the completion of these studies, and Miss Joan Rowe, for technical assistance.

REFERENCES

1. Romansky, M. J., and Rittman, G. E.: Penicillin: Prolonged Action in Beeswax-Peanut Oil, New England J. Med. **233**: 577, 1945.
Dept. (no. 81) p. 43, 1944; abstr., A Method of Prolonging the Action of Penicillin, Science **100**: 196, 1944.
2. Romansky, M. J., and Rittman, G. E.: Penicillin Blood Levels for Twenty-Four Hours Following a Single Intramuscular Injection of Calcium Penicillin in Beeswax and Peanut Oil, New England J. Med. **233**: 577, 1945.
3. Kirby, W. M. M., Leifer, W., Martin, S. P., Rammelkamp, C. H., and Kinsman, J. M.: Intramuscular and Subcutaneous Administration of Penicillin in Beeswax-Peanut Oil, J. A. M. A. **129**: 940, 1945.

4. Robertson, D. F.: Personal communication.
5. Rammelkamp, C. H.: Method for Determining Concentration of Pencillin in Body Fluids and Exudates, Proc. Soc. Exper. Biol. & Med. **51**: 95, 1942.
6. Kirby, W. M. M., Martin, S. P., Leifer, W., and Kinsman, J. M.: Maintenance of Therapeutic Blood Concentrations of Penicillin for Twenty-Four Hours Following Single Injections of Penicillin-Beeswax-Peanut Oil Mixtures, J. LAB. & CLIN. MED. **31**: 313, 1946.
7. Romansky, M. J., Murphy, R. J., and Rittman, G. E.: Single Injection Treatment of Gonorrhea With Penicillin in Beeswax-Peanut Oil; Results in 175 Cases, J. A. M. A. **128**: 404, 1945.
8. Van Slyke, C. J., and Heller, J. R., Jr.: Treatment of Gonorrhea by a Single Intramuscular Injection of Penicillin-Oil-Beeswax; a Cooperative Study of 1,060 Cases, Ven. Dis. Inform. **26**: 98, 1945.
9. Graham, W. E., Greenblatt, R. B., and Cannefax, G. R.: Accelerated Methods of Treating Gonorrhea in the Female With Penicillin-Wax-Oil Mixtures, Ven. Dis. Inform. **26**: 105, 1945.
10. Leifer, W., Martin, S. P., and Kirby, W. M. M.: The Treatment of Gonococcal Urethritis With Single Injections of Penicillin-Beeswax-Peanut Oil Mixtures, New England J. Med. **233**: 583, 1945.
11. Chinn, B. D., Olansky, S., and Murphy, I. G.: The Treatment of Gonorrhea by a Single Injection of a Penicillin-Oil-Beeswax Mixture, Med. Ann. District of Columbia **15**: 62, 1946.
12. Geiger, A. J., and Goerner, J. R.: The Treatment of Subacute Bacterial Endocarditis With Penicillin in Peanut Oil and Beeswax, New England J. Med. **235**: 285, 1946.

PHARMACODYNAMIC EFFECT IN MAN OF STREPTOMYCIN CONTAINING A HISTAMINE-LIKE FACTOR

WILLIAM L. HEWITT, M.D., AND JOHN J. CURRY, M.D.
BOSTON, MASS.

IN A systematic study of the pharmacodynamic effects of streptomycin in animals, it has been demonstrated that the reactions to the pure compound differ from those of preparations which contain impurities.[1] Administration to man of streptomycin containing these impurities has resulted in severe reactions and in two instances death has occurred.[2] Solutions containing one of these contaminants produce a sudden fall in arterial blood pressure, peripheral vasodilatation, and inhibition of water diuresis when injected into rabbits. However, when the solutions are incubated with histaminase prior to injection, these reactions do not occur.[3] Therefore, it has been considered generally that the impurity is histamine. Herein are presented additional data concerning the pharmacodynamic effects of this impurity of streptomycin solutions in man. For convenience, streptomycin solutions containing the histamine-like contaminating substance will be designated H-streptomycin and regular commercial streptomycin will be designated C-streptomycin.*

MATERIALS AND METHODS

Young adults who were in the terminal phases of penicillin therapy for primary and secondary syphilis but who were otherwise in good health and gave no personal or familial history of allergic disorder were used as subjects. H-streptomycin was dissolved in sterile distilled water in concentration of 100 mg. per cubic centimeter for intramuscular administration and further diluted to 5 mg. per cubic centimeter in physiologic saline for intravenous use. Control observations were made using C-streptomycin prepared for administration in a similar manner. Benadryl was dissolved in physiologic saline in a concentration of 30 mg. per 50 c.c. for intravenous use.

H-streptomycin was then injected intramuscularly in 100 to 500 mg. doses and intravenously in 50 mg. doses, and observations were made with special attention to peripheral vasodilatation, blood pressure, pulse rate, headache, and palpitation. The effectiveness of benadryl, a potent antihistamine substance, in altering these reactions was next observed by administering a dose of 10 mg. of benadryl intravenously five to seven minutes prior to the intramuscular injection of 500 mg. of H-streptomycin. In other subjects a dose of 10 mg.

From the Evans Memorial, Massachusetts Memorial Hospitals, and the Department of Medicine, Boston University School of Medicine.

This work was supported in part by a grant from The Upjohn Company, Kalamazoo, Mich.

Received for publication, Nov. 1, 1946.

*We are indebted to E. R. Squibb & Sons, New Brunswick, N. J., for supplying both lots of streptomycin. Manufacturers' tests and Food and Drug Administration regulations now prevent lots of streptomycin containing the histamine-like factor from reaching commercial channels.

of benadryl was given intravenously one minute after the intravenous injection of 50 mg. of H-streptomycin was begun and when the histamine-like reaction to it was well established. Comparatively small doses of benadryl were used because our supply of the drug was limited.

The effect of H-streptomycin on the gastric secretion was studied in nine subjects by analysis of the volume and free and total acidity of the gastric contents prior to and after the intramuscular injection of 500 mg. of H-streptomycin.

In two asthmatic subjects the effect of 50 mg. of H-streptomycin on the vital capacity was studied by methods previously described.[4, 5] Control observations were made using histamine base and C-streptomycin. The effectiveness of benadryl in preventing the reduction in the vital capacity which followed the injection of H-streptomycin and histamine base was then determined.

RESULTS AND COMMENT

Intravenous administration of 50 mg. of H-streptomycin produced a characteristic response in all of twenty patients tested. This consisted of a sensation of facial or generalized warmth, palpitation, throbbing headache, and objective evidence of peripheral vasodilatation by observation of facial suffusion. No notable alteration occurred in the pulse rate, respiration, or blood pressure, as determined by the usual clinical methods. Intramuscular administration of doses up to 500 mg., however, produced these changes in only 48 per cent of twenty-three individuals tested with the largest dose, 500 mg., and the reactions when present were frequently less severe than following intravenous administration of the drug (Table I). In seven subjects, when the intravenous administration of H-streptomycin was initiated and the characteristic response was evident, 10 mg. of benadryl were injected intravenously in the opposite arm. A prompt and marked amelioration of symptoms followed, and rapid blanching of the facial suffusion was noted despite the continued administration of the remainder of the 50 mg. dose of H-streptomycin. A dose of 10 mg. of benadryl was given intravenously to six subjects who previously showed sensitivity to intramuscular H-streptomycin, and five to seven minutes later the intramuscular administration of 500 mg. of H-streptomycin failed to produce the characteristic response.

A stimulating effect of H-streptomycin on the gastric secretion which closely resembles a histamine response is shown in Table II. A significant increase in free acidity and an increased total volume occurred in at least seven of the nine subjects studied.

In two asthmatic subjects it was previously shown that a quantitatively similar decrease in vital capacity followed the intravenous injection of identical amounts of histamine. Therefore, in Subject B. R., after a control injection of 50 mg. of C-streptomycin administered intravenously had produced no notable effect, a dose of 50 mg. of H-streptomycin was given by the same route, and vital capacities were recorded at intervals. The reduction in vital capacity that followed was similar to the reduction produced by the intravenous administration of 0.01 mg. histamine base. The protection afforded

TABLE I. EFFECT OF PARENTERAL ADMINISTRATION OF STREPTOMYCIN CONTAINING HISTAMINE-LIKE FACTOR

NUMBER OF SUBJECTS	DOSE (MG.)	ROUTE OF ADMINISTRATION	HEADACHE	PALPITATION	FLUSH	NO REACTION
20	50	I.V.	12	3	19	0
6	100	I.M.	0	0	1	5
21	200	I.M.	2	0	3	17
23	500	I.M.	3	0	9	12

TABLE II. EFFECT OF STREPTOMYCIN CONTAINING HISTAMINE-LIKE FACTOR ON GASTRIC SECRETION

	VOLUME		FREE HYDROCHLORIC ACID	
SUBJECT	BEFORE H-STREPTOMYCIN (C.C.)	AFTER H-STREPTOMYCIN (C.C.)	BEFORE H-STREPTOMYCIN (UNITS)	AFTER H-STREPTOMYCIN (UNITS)
H. T.	36	175	17	97
J. B.	33	75	23	35
G. H.	20	30	0	26
E. R.	15	100	15	60
L. S.	10	36	0	55
J. J.	14	58	0	82
C. N.	50	55	0	90
E. H.	15	40	0	102
R. H.	32	42	0	65

by 30 mg. of benadryl given intravenously against the reduction in vital capacity produced by 50 mg. of H-streptomycin and 0.01 mg. of histamine base likewise was similar. However, when 4 mg. of acetyl-β-methylcholine chloride was injected intramuscularly thirty-four minutes after the benadryl was given, a pronounced decrease in vital capacity occurred, and epinephrine was necessary to afford relief from the induced asthmatic-like attack (Fig. 1).

In Subject J. D., after administration of 50 mg. of C-streptomycin had produced no notable effect, 50 mg. of H-streptomycin were injected intravenously, and the vital capacity was recorded at intervals. The reduction in vital capacity that followed was similar to the reaction due to the intravenous injection of 0.02 mg. of histamine base. As in the previous case, 30 mg. of benadryl administered intravenously afforded a similar protection against the reduction in vital capacity due to 50 mg. of H-streptomycin and 0.02 mg. of histamine given by vein. The reduction in vital capacity that followed the intramuscular injection of 1.0 mg. of acetyl-β-methylcholine chloride was not affected by the administration of benadryl (Fig. 2). In these two asthmatic subjects the reaction of the tracheobronchial tree to H-streptomycin and to histamine was similar. The protection afforded by 30 mg. of benadryl against H-streptomycin and histamine was likewise similar. The failure of benadryl in the dosage used to protect against the reduction in vital capacity due to acetyl-β-methylcholine chloride appears to indicate that the streptomycin contaminant is not related to acetyl-β-methylcholine chloride. Since it has been shown previously that under certain circumstances quantitatively similar amounts of bronchoconstriction can be produced by identical amounts of histamine, it may be possible to determine quantitatively the amount of histamine-like contaminant present in streptomycin solutions by this method.

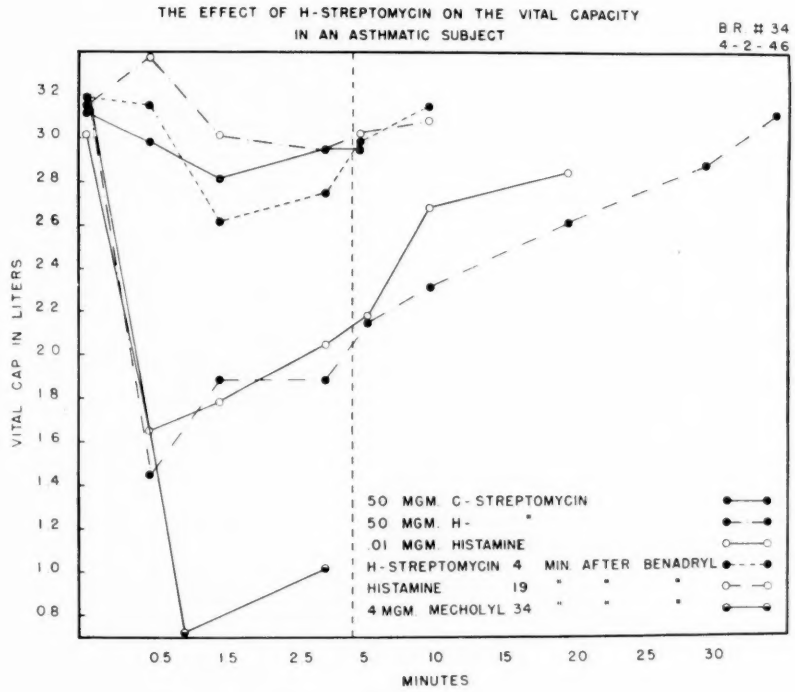

THE EFFECT OF H-STREPTOMYCIN ON THE VITAL CAPACITY
IN AN ASTHMATIC SUBJECT
B.R. # 34
4-2-46
VITAL CAP IN LITERS
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.5
1.5
2.5
5
10
15
20
25
30
MINUTES
50 MGM. C-STREPTOMYCIN
50 MGM. H- "
.01 MGM. HISTAMINE
H-STREPTOMYCIN 4 MIN. AFTER BENADRYL
HISTAMINE 19 " " "
4 MGM. MECHOLYL 34 " " "

Fig. 1.

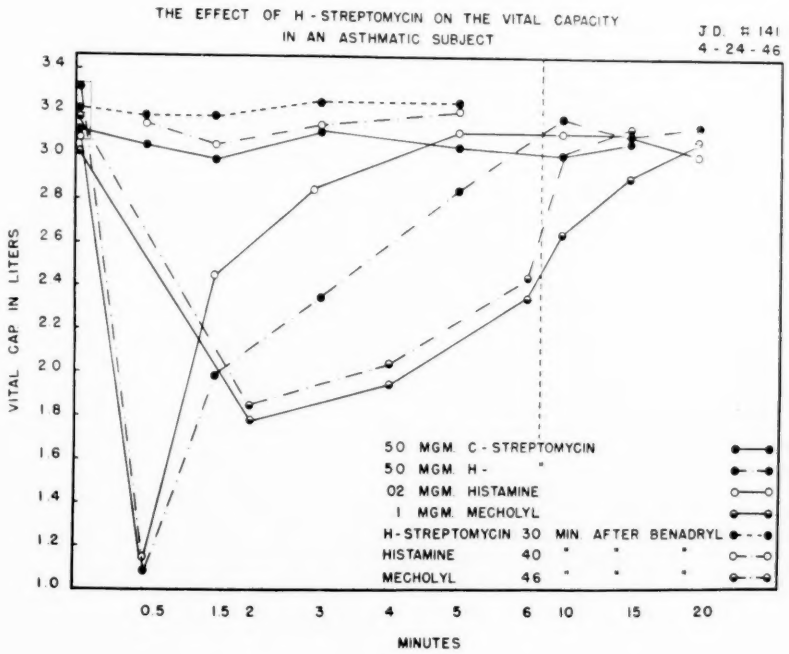

THE EFFECT OF H-STREPTOMYCIN ON THE VITAL CAPACITY
IN AN ASTHMATIC SUBJECT
J.D. # 141
4-24-46
VITAL CAP IN LITERS
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.5
1.5
2
3
4
5
6
10
15
20
MINUTES
50 MGM. C-STREPTOMYCIN
50 MGM. H- "
.02 MGM. HISTAMINE
1 MGM. MECHOLYL
H-STREPTOMYCIN 30 MIN. AFTER BENADRYL
HISTAMINE 40 " " "
MECHOLYL 46 " " "

Fig. 2.

SUMMARY

1. Throbbing headache, palpitation, and peripheral vasodilatation were observed uniformly in twenty young adult subjects following the intravenous administration of 50 mg. of streptomycin containing a histamine-like factor, but a similar histamine-like reaction was observed in only 48 per cent of twenty-three subjects following intramuscular administration of 500 mg. of the same material.

2. Small doses of benadryl given intravenously were effective in ameliorating these reactions to H-streptomycin.

3. In nine fasting young adult subjects, 500 mg. of streptomycin containing a histamine-like factor, administered intramuscularly, produced a significant increase in total volume of gastric secretion and the amount of free acid present.

4. In one asthmatic subject the intravenous administration of 50 mg. of H-streptomycin and 0.01 mg. of histamine base produced a similar degree of reduction in the vital capacity which was almost completely prevented by 30 mg. of benadryl given intravenously. In another subject essentially similar results were achieved when 0.02 mg. of histamine base was administered. Benadryl in the dosage used afforded no notable protection against the reduction in vital capacity due to acetyl-β-methylcholine chloride.

5. These studies in man furnish further evidence that one of the contaminating substances of impure solutions of streptomycin is histamine.

REFERENCES

1. Molitor, H., Graessle, D. E., Kuna, S., Mushett, C. W., and Silber, R. H.: Some Toxicological and Pharmacological Properties of Streptomycin, J. Pharmacol. & Exper. Therap. **86**: 151-173, 1946.
2. Editorial: Toxicity of Streptomycin, J. A. M. A. **131**: 745-746, 1946.
3. Editorial: Streptomycin, J. A. M. A. **130**: 939-940, 1946.
4. Curry, J. J.: The Action of Histamine on the Respiratory Tract in Normal and Asthmatic Subjects, J. Clin. Investigation **25**: 785-791, 1946.
5. Curry, J. J.: The Effect of Antihistamine Substances and Other Drugs on Histamine Bronchoconstriction in Asthmatic Subjects, J. Clin. Investigation **25**: 792-799, 1946.

HISTAMINE ANTAGONISTS

VI. Comparative Antihistaminic Activity of Some Ethylenediamine Drugs in the Guinea Pig

Sidney Friedlaender, M.D., Detroit, Mich., and Samuel M. Feinberg, M.D., and Alan R. Feinberg, M.D., Chicago, Ill.

RAPID progress in the field of histamine antagonists has resulted in the development of several synthetic compounds which have proved successful to some degree in alleviating certain allergic manifestations in man. All of these substances are chemically and pharmacologically related. Among the first to be introduced in this country was benadryl, a benzhydryl ether of β-dimethylaminoethanol.[1] In France, N′ phenyl-N′ benzyl-N-dimethylethylenediamine (antergan) has been used clinically since 1942.[2] A search for more potent and less toxic compounds led to the development of two other substances, pyribenzamine[3] and neoantergan.[4] The former differs from antergan only by substitution of the pyridil for the phenyl ring, while neoantergan differs from pyribenzamine only by the addition of a methoxy group on the benzyl ring (Table I). A comparative study of the antihistaminic activity of these four compounds was undertaken, in order to serve as a basis for future studies in anaphylaxis.*

EXPERIMENTAL

The guinea pig responds to intravenous doses of histamine with characteristic bronchospasm and asphyxia difficult to distinguish from that observed in anaphylactic shock. The effect is rapid in onset, and death from fatal doses occurs within a few moments. Post-mortem examination reveals the characteristic pale emphysematous lungs in each instance. Unlike anaphylaxis, nonfatal histamine shock does not confer immunity to subsequent injections of histamine. In the present experiments the histamine was injected into the penile veins of adult male guinea pigs. The intracardiac route was avoided because of the possibility of fatalities from the direct action of histamine on the coronary circulation or other cardiac tissues.

From preliminary experiments[5] we determined that 0.4 mg. per kilogram of histamine,† calculated in terms of the base, killed all control animals. Shock occurred in all guinea pigs from 0.3 mg. per kilogram but terminated fatally in only 50 per cent. All animals survived a dose of 0.1 mg. per kilogram of histamine. In order to determine as objectively as possible the degree of antihistaminic activity of each of these compounds, the amount of histamine neces-

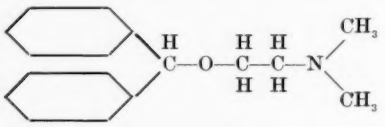
From the Department of Medicine, Division of Allergy, Northwestern University Medical School, Chicago, Ill.

Received for publication, Nov. 4, 1946.

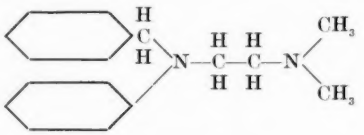
*Benadryl was supplied by Parke, Davis & Company, Detroit, Mich.; pyribenzamine by Ciba Pharmaceutical Products, Inc., Summit, N. J.; antergan and neoantergen by Rhone-Poulenc, Paris, France.

†Histamine phosphate was furnished by Burroughs Wellcome & Co. (U. S. A.) Inc., New York, N. Y.

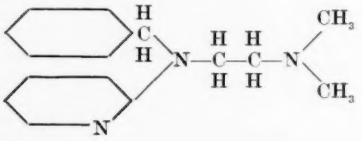
TABLE I



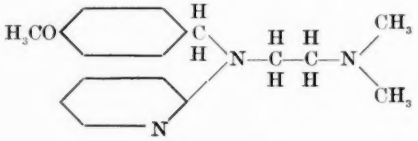
Benadryl



Antergan



Pyribenzamine



Neoantergan

sary to produce 100 per cent mortality in a group of guinea pigs receiving a standard dose of the drug was determined. In each instance the standard protective dose was 3 mg. per kilogram and was administered intraperitoneally fifteen minutes prior to the intravenous injection of histamine. It was previously determined that none of the solutions produced fatal toxic symptoms on intraperitoneal injection in doses below 50 mg. per kilogram. Animals surviving histamine shock were not used again for at least forty-eight hours.

RESULTS (TABLES II AND III)

In those animals protected with 3 mg. per kilogram of benadryl, the dose fatal to all controls (0.4 mg. per kilogram of intravenous histamine) produced only a 29 per cent mortality. Two milligrams per kilogram were necessary to kill all benadryl-protected animals. Antergan gave approximately the same amount of protection, 2.4 mg. per kilogram of histamine being required for the 100 per cent fatal dose in animals receiving this drug. Equivalent doses of pyribenzamine afforded more protection against histamine than either benadryl or antergan. The animals receiving pyribenzamine exhibited little evidence of shock from doses of histamine as high as 2.0 mg. per kilogram. The majority recovered from histamine shock produced by 10 mg. per kilogram, while a dose of 15.0 mg. per kilogram was found fatal for all guinea pigs in this group. Neoantergan, which is only slightly different in chemical structure from pyribenzamine, conferred even greater protection on some animals. While 10 mg. per kilogram of histamine produced approximately the same mortality in this group as in the pyribenzamine-protected guinea pigs, more than half of the neoantergan animals were able to survive doses of 25.0 mg. per kilogram. Fifty milligrams per kilogram of histamine were found to be the 100 per cent fatal dose in this group. On the basis of the number of lethal doses of histamine required to produce 100 per cent mortality in animals protected with a standard

TABLE II. PROTECTIVE EFFECT OF BENADRYL AND ANTERGAN

HISTAMINE I.V. [MG. (BASE)/KG.]	CONTROL GROUP (MORTALITY) Total deaths / Total used	(%)	GROUP RECEIVING BENADRYL, 3 MG./KG. (MORTALITY) Total deaths / Total used	(%)	GROUP RECEIVING ANTERGAN, 3 MG./KG. (MORTALITY) Total deaths / Total used	(%)
.03 to 0.1	0/3	0				
0.2	2/6	33				
0.3	3/6	50	0/5	0		
0.4	10/10	100	2/7	29	0/10	0
0.8 to 1.6			2/6	33	6/10	60
2.0			6/6	100	4/5	80
2.4					9/9	100

TABLE III. PROTECTIVE EFFECT OF PYRIBENZAMINE AND NEOANTERGAN

HISTAMINE I.V. [MG. (BASE)/KG.]	CONTROL GROUP (MORTALITY) Total deaths / Total used	(%)	GROUP RECEIVING PYRIBENZAMINE, 3 MG./KG. (MORTALITY) Total deaths / Total used	(%)	GROUP RECEIVING NEOANTERGAN, 3 MG./KG. (MORTALITY) Total deaths / Total used	(%)
.03 to 0.1	0/3	0				
0.2	2/6	33				
0.3	3/6	50				
0.4	10/10	100	0/6	0	0/5	0
0.18 to 1.6			0/5	0		
2.0			0/4	0	0/6	0
2.4 to 4.8			1/6	16		
5.0			2/6	33	0/10	0
5.2 to 6.4			1/5	25		
6.8 to 9.0			1/5	25		
10.0			2/5	40	9/24	37
11.0 to 13.0			5/8	62		
15.0			10/10	100	9/19	47
20.0					4/9	44
25.0					5/11	45
30.0					7/10	70
40.0					8/10	80
50.0					10/10	100

TABLE IV. ANTIHISTAMINIC ACTIVITY

3 MG./KG. OF PROTECTIVE DRUG	NUMBER OF LETHAL DOSES OF HISTAMINE NECESSARY TO PRODUCE 100% MORTALITY
Benadryl	5
Antergan	6
Pyribenzamine	37
Neoantergan	125

dose of each drug, pyribenzamine would thus appear to be approximately 6 to 7 times more active than either benadryl or antergan, while neoantergan would possess almost 3.5 times the activity of pyribenzamine (Table IV).

DISCUSSION

The protective effect of these agents against the lethal effects of histamine is striking and indicates a great degree of specific antagonism. Various investigators have reported that these compounds also exert marked anti-

anaphylactic activity.[2, 3, 5-7] This action together with the beneficial effects observed in certain allergic disorders is in accord with the theory that histamine plays an important role in anaphylaxis and allergy. In our own experiments we also found these drugs capable of modifying anaphylaxis in the guinea pig. Preliminary studies did not show a marked difference in the antianaphylactic activity of these compounds comparable to the variation exhibited in histamine shock. Further experiments are being carried out in order to clarify, if possible, this apparent discrepancy.

SUMMARY

A comparison was made of four antihistamine substances, benadryl, pyribenzamine, antergan, and neoantergan. All compounds afforded some protection to guinea pigs against the fatal effects of intravenous histamine. Five to six times the LD 100 of histamine was necessary to kill all animals protected with 3 mg. per kilogram of benadryl or antergan. The same dose of pyribenzamine was able to protect some animals against as much as thirty-five lethal doses, while neoantergan protected some animals against as much as three times this amount of histamine.

REFERENCES

1. Loew, E. R., Kaiser, M. E., and Moore, V.: Synthetic Benzhydryl Alkamine Ethers Effective in Preventing Fatal Experimental Asthma in Guinea Pigs Exposed to Atomized Histamine, J. Pharmacol. & Exper. Therap. **83**: 120, 1945.
2. Halpern, B. N.: Experimental Study of Synthetic Antihistaminic Substances: Chemotherapeutic Trials in Allergic States, J. de méd. de Lyon **23**: 409, 1942.
3. Mayer, R. L., Huttrer, C. P., and Scholz, C. R.: Antihistamine and Antianaphylactic Activity of Some *a*-pyridino-ethylenediamines, Science **102**: 93, 1945.
4. Bovet, D., Horclois, R., and Walthert, F.: Antihistamine Properties of N-p-methoxybenzyl-N-dimethylaminoethyl *a* amino-pyridine, Compt. rend. Soc. de biol. **138**: 99, 1944.
5. Friedlaender, S., Feinberg, S. M., and Feinberg, A. R.: Histamine Antagonists. V. Comparative Study of Benadryl and Pyribenzamine in Histamine and Anaphylactic Shock, Proc. Soc. Exper. Biol. & Med. **62**: 65, 1946.
6. Loew, E. R., and Kaiser, M. E.: Alleviation of Anaphylactic Shock in Guinea Pigs With Synthetic Benzhydryl Alkamine Ethers, Proc. Soc. Exper. Biol & Med. **58**: 235, 1945.
7. Wells, J. A., Morris, H. C., and Dragstedt, C. A.: Modification of Anaphylaxis by Benadryl, Proc. Soc. Exper. Biol. & Med. **61**: 104, 1946.

UNUSUAL INSTANCES OF AURICULAR FLUTTER

S. F. Aronson, M.D., and L. N. Katz, M.D.
Chicago, Ill.

THE finding of either 1:1 or persistent 3:1 A-V conduction in auricular flutter is so rare that their occurrence merits attention. Two case histories, each illustrating one of these mechanisms electrocardiographically, are presented.

CASE REPORTS

Case 1.—The first case is that of a 42-year-old white man with a bronchogenic carcinoma and extensive metastases which caused almost complete obstruction of the esophagus. A gastrostomy was performed to relieve the effects of compression. The patient became progressively weaker during his hospital stay. On the evening of the forty-ninth postoperative day, the heart rate became rapid (160 beats per minute) and irregular with a pulse deficit. The rate continued to increase until it was too rapid to be accurately counted. The patient's body became covered by a cold and clammy perspiration, the skin developed a cadaveric hue, and loud bubbling râles could be heard throughout the chest. He lost consciousness and the blood pressure could not be obtained. Oxygen therapy was immediately instituted and an electrocardiogram was taken (Fig. 1). This showed a ventricular rate of 296 beats per minute. Deep pressure applied over the right and left carotid sinuses had no effect on the ventricular rate. About three hours after the onset of the rapid heart rate, 0.48 Gm. of quinidine was given intramuscularly with massage and heat over the injection site to aid in absorption. Ten minutes later, one of the house physicians in attendance noted that the heart rate counted at the apex was 148 beats per minute and regular, and "flutter waves" at twice this rate were visible in the neck veins. The patient regained consciousness and responded to questioning. The slower ventricular rate persisted until the patient's death, which occurred several hours later. The slowing of the heart rate so soon after the intramuscular injection of the quinidine naturally raises doubt that it was due to this drug. Unfortunately, an electrocardiogram demonstrating the change in the heart rate was not obtained, but because the slower rate was exactly one-half that of the faster one and because of the visible auricular waves in the neck veins at a rate of 296, it was felt that the mechanism had converted from auricular flutter with 1:1 conduction to one with 2:1 conduction.

Discussion.—Some authors[1-3] have referred to several of the difficulties which may be encountered in distinguishing auricular flutter from the paroxysmal tachycardias. However, the gradual onset of the arrhythmia, the extremely rapid and regular ventricular rate (296 beats per minute), the absence of electrical quiescence during the auricular cycles, the normal configuration of the QRS complexes, and the clinical observation of the halving of the ventricular rate from 296 to 148 beats per minute with flutter waves visible in the neck veins established the diagnosis of an auricular flutter with 1:1 conduction in this case.

From the Cardiovascular Department, Michael Reese Hospital.
Aided by the A. D. Nast Fund for Cardiovascular Research.
The department is supported in part by the Michael Reese Research Foundation.
Received for publication, Nov. 15, 1946.

The occurrence of transmission of every impulse to the ventricles is extremely rare in auricular flutter. In 1933, Bedell[4] was able to find only twenty-four such cases reported in adults. This is explained by the normally long absolute refractory phase in the A-V node which interferes with the transmission of the frequently recurring auricular impulses, which in auricular

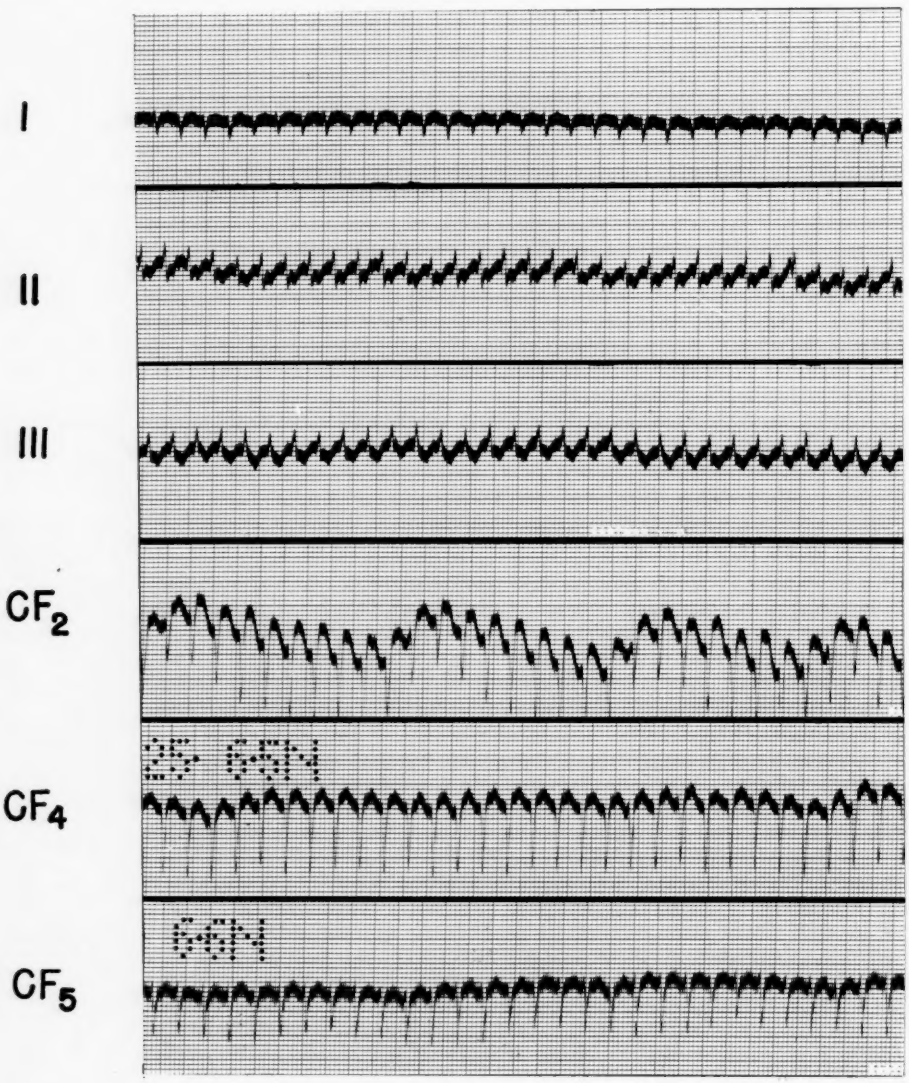


Fig. 1.

flutter have a rate usually between 250 and 400 beats per minute. Only when some circumstance abbreviates the duration of the refractory phase of the A-V node can every impulse be transmitted. Abbreviation of the refractory phase of the A-V node may occur as a result of injury or during sympathetic

stimulation (excitement, adrenalin). Usually this occurs[5, 6] when the flutter rate is slower than 250 beats per minute. Such slowing of the auricular flutter rate is best exemplified by the exhibition of quinidine, whereby the auricular rate may even be slowed to below 200 impulses per minute.

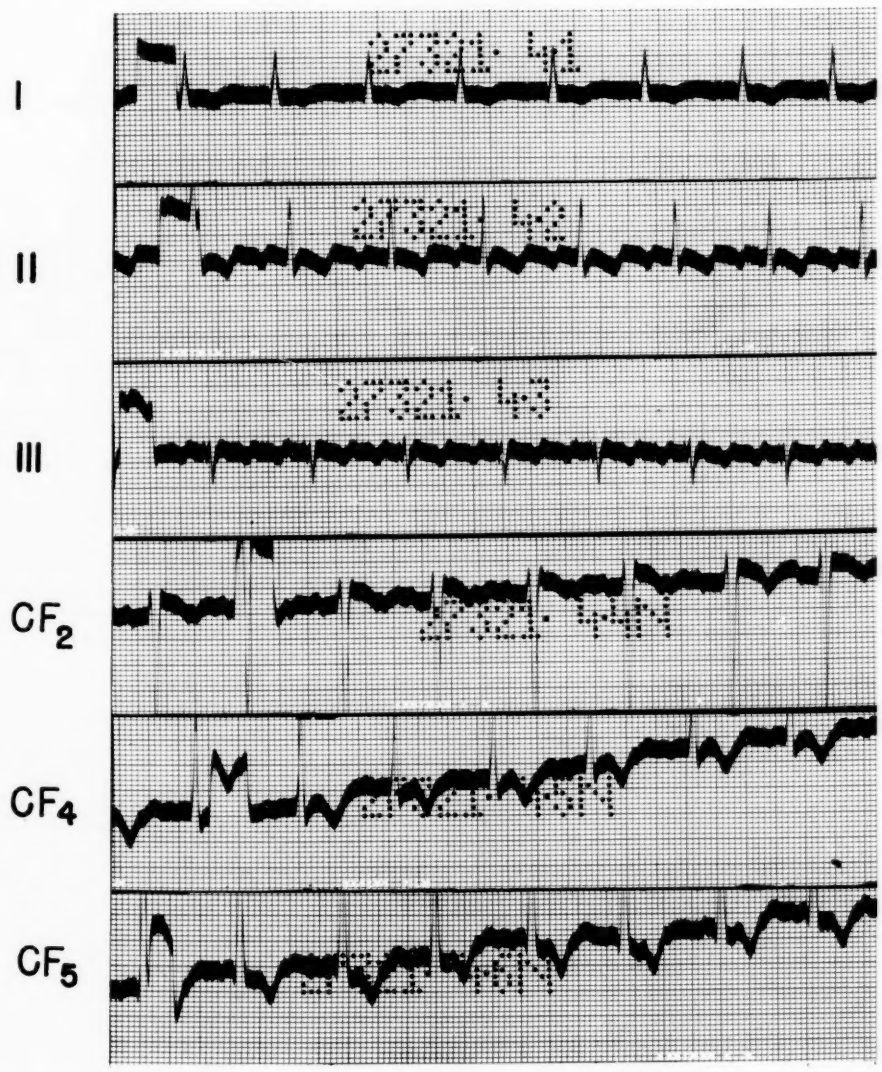


Fig. 2.

The other unusual feature in this case is the fact that 1:1 conduction occurred with an auricular rate of almost 300 making the ventricular rate itself rapid. Such rapid ventricular rates are rare. In 1937, a survey of the literature by Lyon[7] revealed seventeen reported cases with a ventricular rate of over 280 beats per minute. Of these seventeen cases twelve were accompanied by graphic evidence, and of these latter only four (including a 14-

year-old boy) were in adults. Edeiken,[8] in 1943, reported the case of a 46-year-old woman in whom, twenty-two days after an acute myocardial infarction, paroxysms of tachycardia (either supraventricular or 1:1 auricular flutter) occurred with ventricular rates of 310 and 303 beats per minute. Ours is therefore another instance of ventricular tachycardia over 280 which was due to 1:1 auricular flutter.

CASE 2.—This 52-year-old colored woman entered the hospital because of orthopnea, dyspnea on exertion, and swelling of the ankles for the six months prior to admission. An electrocardiogram revealed auricular flutter with a 2:1 A-V conduction. With the institution of therapy, the patient improved clinically. Other electrocardiograms showed persistent flutter with 2:1 and 4:1 A-V conduction and occasional ventricular premature systoles, the auricular rate being about 300 beats per minute. The fourth record (Fig. 2) revealed the very rare phenomenon of auricular flutter with a regular ventricular rate and a persistent 3:1 A-V conduction. Several days after this last tracing, oral quinidine, 0.2 Gm. every two hours and a total dosage of 2.8 Gm., was given following an initial test dose. The auricular flutter persisted, so after a rest period of three days quinidine therapy was again instituted. Within a period of thirty hours the patient received a total of 6 Gm. of quinidine which was divided into three separate courses. Careful check was made for evidence of cinchonism; frequent cardioscopic examinations were made, and an electrocardiogram was taken between each course. Fig. 3*A* shows the conversion of the flutter mechanism to a sinus rhythm with bigeminy (ventricular premature systoles). The bigeminy may have been due, in part at least, to digitalis (0.2 mg. digitaline nativelle daily) which the patient received throughout the period of quinidine administration. All medication was discontinued after the bigeminal rhythm was found, and an electrocardiogram taken forty-eight hours later showed a sinus rhythm with only an occasional ventricular premature systole (Fig. 3*B*).

Discussion.—Occasional cycles with 3:1 conduction in cases of auricular flutter with an irregular ventricular rate are not uncommon. However, the occurrence in auricular flutter of a persistent 3:1 conduction is extremely rare.[6, 9] Thus, this case is the first one in which this phenomenon has been found in over 30,000 electrocardiograms taken at the Michael Reese Hospital. The very regular, saw-toothed flutter waves, occurring at a rate of 270 cycles per minute, are best seen in Leads II and III of Fig. 2. Closer inspection will disclose their presence in the other leads as well. We are aware of only three such previous cases on record.[10-12] It has been postulated that 2:1 A-V conduction in auricular flutter is due to a focal interference which prevents every second auricular impulse from stimulating the ventricles; 4:1 A-V conduction would occur if a second block existed which would allow only one-half of the impulse that had penetrated the first block from reaching the ventricles. This would readily explain the relative frequency of 2:1 A-V conduction and the less common occurrence of 4:1 A-V conduction. However, this theory cannot account for the existence of a persistent 3:1 A-V conduction. The presence, even on occasion, of persistent 3:1 conduction indicates either that the hypothesis previously stated is erroneous or that a single region of interference may become so protracted as to allow the penetration of only one out of every three beats.

Another interesting feature in this case is the establishment of a bigeminal rhythm after the flutter mechanism was broken (Fig. 3*A*). Finally, in Fig. 3*B*

is illustrated the quinidine effect on the electrocardiogram with marked T-wave inversions (except in Lead III) and the prolongation of electrical systole (0.40 second; normal, 0.29 to 0.37 second).

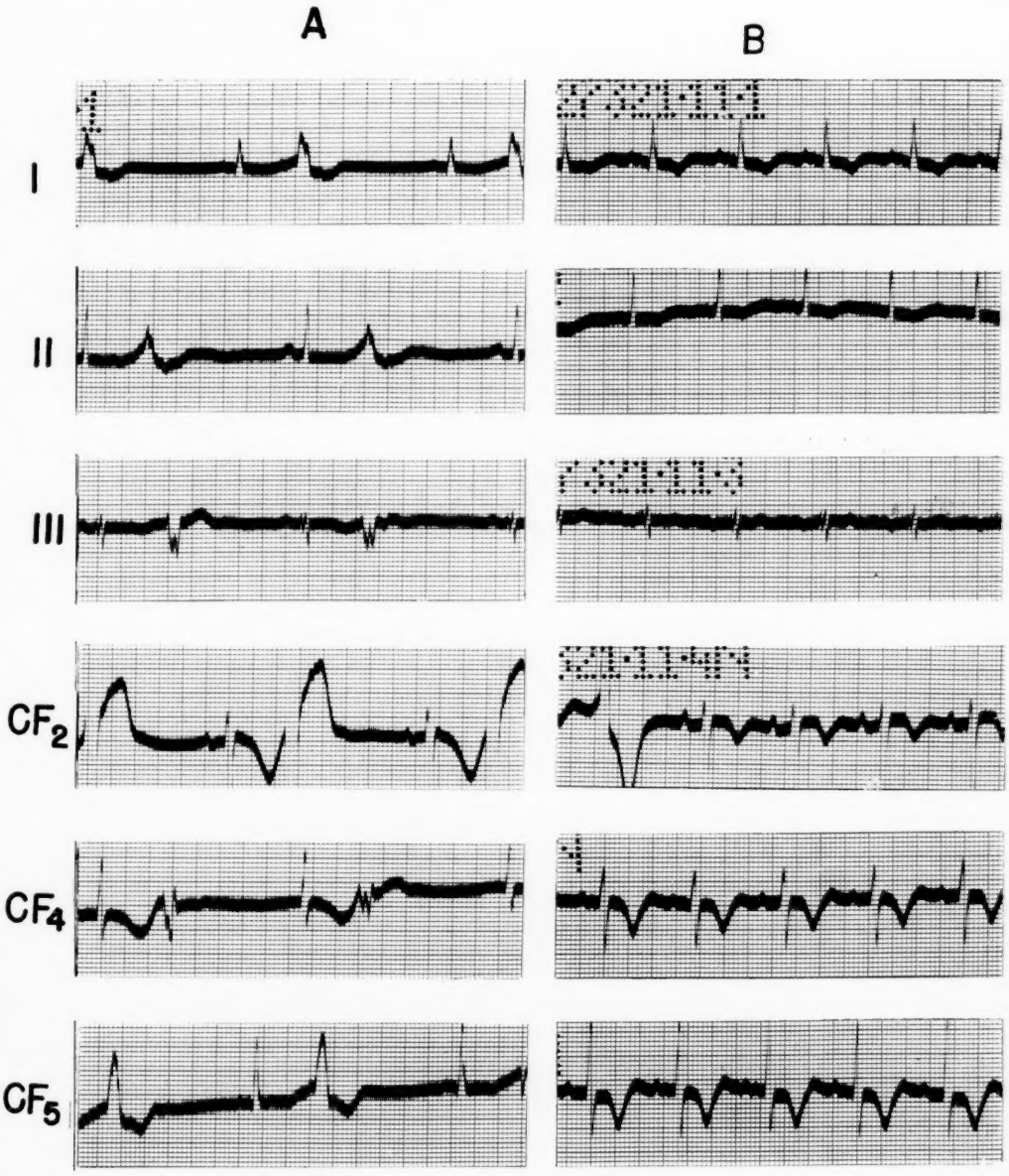


Fig. 3.

SUMMARY

1. A very rare instance of auricular flutter with a 1:1 conduction and a ventricular rate of 296 beats per minute is presented.

2. An even rarer phenomenon is shown, namely, a case of auricular flutter with a persistent 3:1 A-V conduction.

REFERENCES

1. Decherd, G. M., Jr., Herrmann, G. R., and Schwab, E. H.: Paroxysmal Supraventricular Tachycardia With A-V Block, Am. Heart J. **26**: 446, 1943.
2. Evans, W.: The Unity of Paroxysmal Tachycardia and Auricular Flutter, Brit. Heart J. **6**: 221, 1944.
3. Barker, P. S., Wilson, F. N., Johnston, F. D., and Wishart, S. W.: Auricular Paroxysmal Tachycardia With Auriculoventricular Block, Am. Heart J. **25**: 765, 1943.
4. Bedell, C. C.: Auricular Flutter With 1:1 Response, Bull. Johns Hopkins Hosp. **52**: 225, 1933.
5. Pardee, H. E. B.: Clinical Aspects of the Electrocardiogram, ed. 4, New York, 1941, Paul B. Hoeber, Inc., p. 219.
6. Katz, L. N.: Electrocardiography, ed. 2, Philadelphia, 1946, Lea & Febiger, p. 666.
7. Lyon, J. A.: Excessively Rapid Heart Rates. Report of a Case With Autopsy, J. A. M. A. **108**: 1393, 1937.
8. Edeiken, J.: Extreme Tachycardia, With Report of Nonfatal Paroxysms Following Myocardial Infarction, Am. J. M. Sc. **205**: 52, 1943.
9. Ashman, R., and Hull, E.: Essentials of Electrocardiography, ed. 1, New York, 1937, The Macmillan Company, p. 186.
10. Graybiel, A., and White, P. D.: Electrocardiography in Practice, ed. 1, Philadelphia, 1941, W. B. Saunders Company, p. 280.
11. Sigler, L. H.: The Electrocardiogram, New York, 1944, Grune & Stratton, Inc., p. 131.
12. Wenckebach, K. F., and Winterberg, H.: Die unregelmässige Herztätigkeit, Vienna, 1927, Wilhelm Engelmann, p. 170.

EFFECT OF OXYGEN ON MALARIA

An in Vivo Study in Ducks

R. H. Rigdon and Nona B. Varnadoe
Little Rock, Ark.

PATHOLOGIC studies on a child dying from a *Plasmodium falciparum* infection,[1] on monkeys dying from *Plasmodium knowlsi* infection,[2] and on ducks dying from *Plasmodium lophurae* infection[3] showed lesions that were similar to those occurring in shock. It was suggested that they resulted from anoxemia. This anoxemia resulted from the anemia produced by the rapid destruction of red blood cells by the plasmodia. Subsequent studies have shown that the blood of ducks with a severe malarial infection at the time of death may have only 15 to 20 per cent of the normal blood oxygen carrying capacity.[4] Anoxemia in malaria is again manifest when ducks with malaria are placed in a decompression chamber in that they succumb before the non-infected birds. The length of survival in these malaria-infected ducks, when in the presence of decreasing amounts of oxygen, may be correlated with the degree of parasitemia.

The clinical observations of Kean and associates[5, 6] support the role that shock may play in acute malarial infections in man. They presented six cases in support of the opinion that medical shock may develop in patients with algid malaria. In three of their cases there was "hemoconcentration, evidence of an increase in the hematocrits, red blood cell count, and hemoglobin. The blood pressure was lowered and there were other signs of vasomotor collapse. . . . The final evidence for shock in these cases was the response to replacement therapy. Plasma was used in three cases, whole blood transfusion in one, oxygen in two, 'Eschatin' in two, intravenous fluids in five, and epinephrine in five."

Ducks with a severe malarial infection show clinical improvement immediately when given a transfusion of duck blood.[7] The red blood cells replace those destroyed by the parasites; thereby more oxygen can be transferred to the tissue. A study has been made to determine the effect of increasing the amount of oxygen available to ducks to see the effect upon the course of the disease. The results of this study are reported at this time.

METHODS AND MATERIAL

White Pekin ducks 2 to 4 weeks of age were used. Blood from donor birds infected with *P. lophurae* was used to infect the ducks. In each experiment a group of birds from the same hatching were inoculated intravenously with blood from the same donor bird.

From the Department of Pathology, School of Medicine, University of Arkansas.
Research paper No. 598, Journal Series, University of Arkansas.
This work was aided by a grant from the John and Mary R. Markle Foundation.
The oxygen was supplied by the Linde Air Products Company and the anticoagulant Liquaemin by Roche Organon, Incorporated.
Received for publication, Oct. 9, 1946.

The course of the infection was followed by counting the number of parasitized cells per 500 red blood cells. Blood films were stained with a combination of Giemsa's and Wright's stains.

Total red blood cell counts were made on some of the ducks. Haymen's fluid was used as the diluent. Hematocrit readings were obtained by centrifuging 10.0 c.c. of heparinized blood for thirty minutes in Wintrobe tubes. Liquaemin was the anticoagulant and was used in a dilution of 1 to 9.

The oxygen chamber was 60 by 32 by 24 inches in size. It was made from galvanized iron supported by an angle iron frame. The bottom had a 2 inch drop from one end to the other. The top of the chamber was made of glass. The door was located at one end of the chamber. A wire meshed floor was placed 3 inches from the bottom. A 2 inch pipe was put in the bottom of the chamber at its lowest point. Water was run continuously into a container used for the ducks to drink from, while the overflow ran over the bottom of the chamber and out into a floor drain. Two small holes were made through the top of the chamber. A thermometer was kept in one and samples of oxygen were removed through the other.

A tank of oxygen was placed adjacent to one end of the chamber. A regulator was used to control the flow of oxygen. The rubber tube carrying the oxygen entered the chamber through a hole in the top. The concentration of oxygen within the chamber was determined frequently with an oxylizer.* The concentration of oxygen varied during the experiments; usually it was kept at a concentration of 50 to 60 per cent during the first few days of the disease and then increased to 80 to 90 per cent. In some experiments the birds were kept continuously within the oxygen chamber for days, while in others they were removed daily for short intervals to obtain blood for the tests. Samples for hematocrit determination were obtained by cardiac puncture, and the birds were discarded thereafter. Blood for cell counts and films were obtained by puncturing a vessel in either the leg or web of the foot.

EXPERIMENTAL

Variations in Erythrocyte Count of Normal Ducks Kept in Oxygen Chamber.—Six young ducks were kept in the oxygen chamber for two weeks with a 50 to 70 per cent concentration of oxygen. The average red blood cell count in these birds is shown in Fig. 1. During the first five days there was a diminution in the total number of red cells, after which the number rapidly returned to normal levels. The erythrocyte count remained within the range of normal during the twelve subsequent days during which the birds were kept in the oxygen chamber. There was no variation in the red cell count for six days after the ducks were removed from the oxygen chamber.

Effect of Oxygen on Parasitemia in Ducks Infected With P. lophurae.—Twenty ducks 15 days of age were inoculated with malaria and ten were put into the chamber with a concentration of 50 per cent oxygen. The parasitemia was followed in five birds in the oxygen chamber and in five of the group kept in the duck room. The average degree of parasitemia in these two groups of ducks is shown in Fig. 2. The parasitemia is the same in the two groups until the fourth day following which time there is an increase in the number of parasites in the oxygen-treated group when compared with those kept in the duck room. By the sixth day of the disease, eight of the ten ducks with malaria kept in the duck room were dead, while only five of the ten ducks

*The Ohio Chemical & Mfg. Company, Cleveland, Ohio.

with malaria kept in the oxygen chamber were dead. The concentration of oxygen was increased to approximately 75 to 80 per cent on the morning of the sixth day as shown in Fig. 2. The remaining five ducks in the oxygen chamber survived until the morning of the seventh day. These five ducks died within seven minutes following the time they were removed from the oxygen chamber.

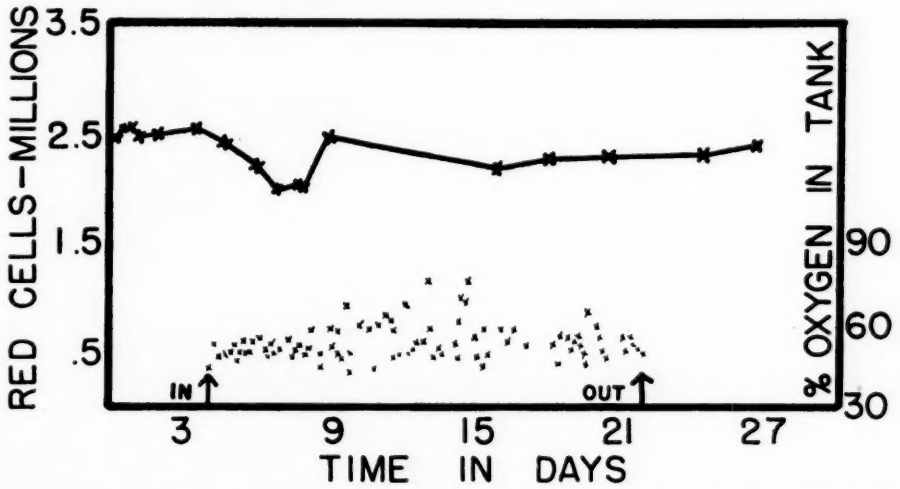


Fig. 1.—Changes which occur in the number of red cells in normal ducks kept in an oxygen chamber.

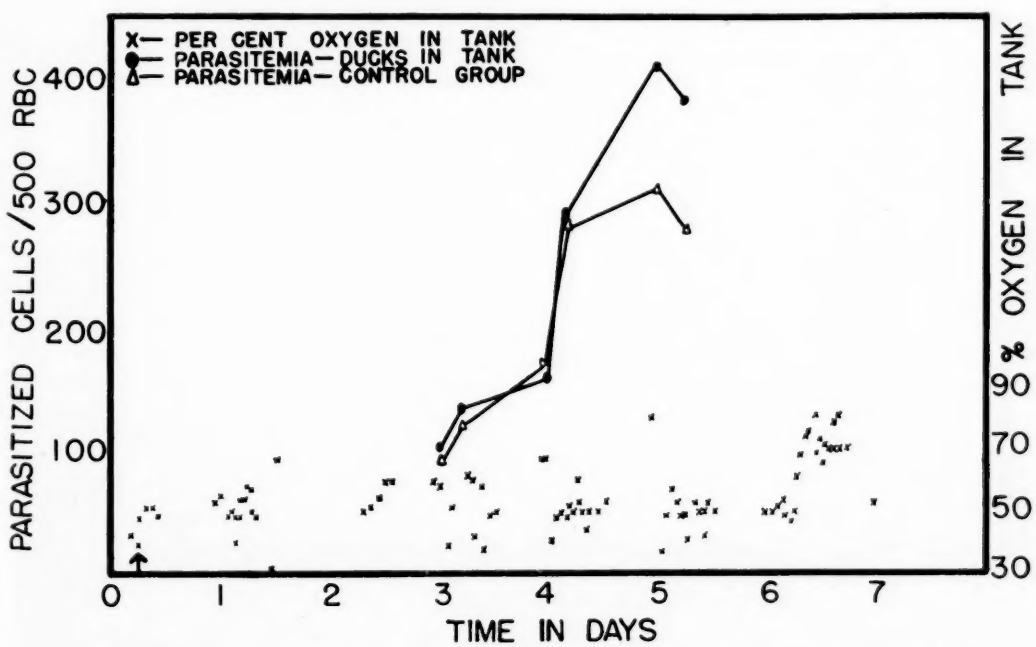


Fig. 2.—The average degree of parasitemia in five ducks kept in the oxygen chamber and of five ducks kept in normal atmosphere. The concentration of oxygen is indicated on the graph.

TABLE I

EXPERIMENTAL DAY	OXYGEN CHAMBER	CONTROLS KEPT IN DUCK ROOM
1	4.1	3.0
2	9.4	8.0
3	16.7	15.6
5 (A.M.)	285.3	127.9
5 (P.M.)	290.0	126.0
6 (A.M.)	325.0	158.0
6 (P.M.)	300.0	137.9
7 (A.M.)	129.0	59.3
7 (P.M.)	95.0	26.0

The effect of oxygen on the parasitemia in ducks was determined in a second experiment in which forty-one birds 16 days of age were used. Twenty of these were put into the oxygen chamber on the fourth day following inoculation. The concentration of oxygen was kept at approximately 50 per cent for twenty-four hours, after which time it was increased to 85 to 90 per cent for the following two days when the experiment was discontinued. The average parasitemia of twenty of these birds was 286 parasitized cells per 500 red blood cells on the fourth day, at the time that the twenty ducks were put into the oxygen chamber. The average parasitemia of ten of these ducks in

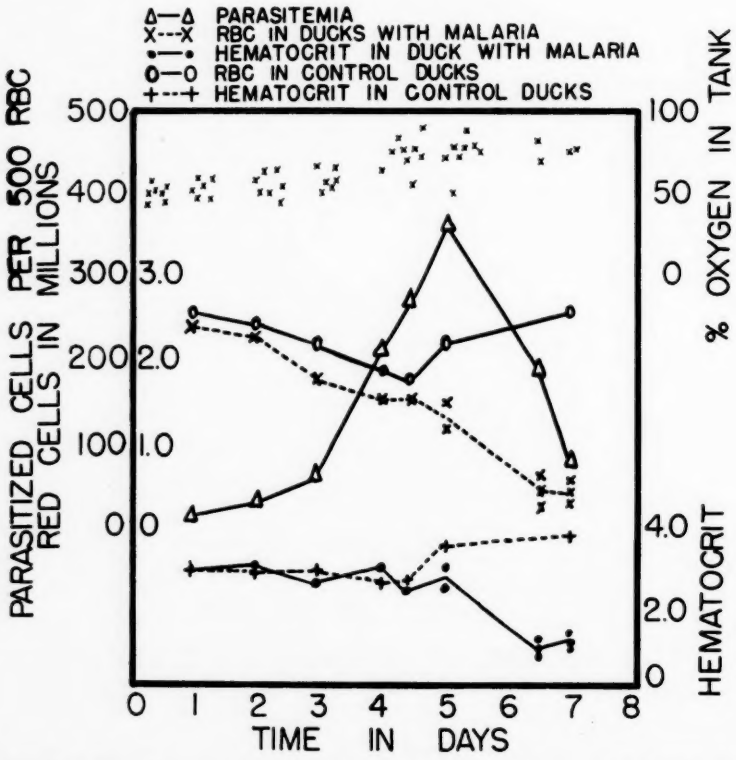


Fig. 3.—The red cell counts, the hematocrit readings, and the parasitemia as observed in ducks infected with *P. lophurae* kept in the oxygen chamber. Two malaria-infected ducks were used to establish each point, except on the sixth day when five birds were used. One normal duck was used to establish the daily values for the controls. A progressive anemia occurred in the malaria-infected birds, while a temporary anemia occurred in the noninfected birds kept under the same environmental conditions.

the oxygen chamber after eighteen hours was 420 parasitized cells per 500 red blood cells, while the average of twenty control ducks was 340 cells. At 9:45 A.M. on the sixth day, eighteen ducks were dead from the group of twenty-one given malaria and kept in the duck room, while only eleven birds were dead from the group of twenty given malaria and put in the oxygen chamber. One of the group removed from the oxygen chamber died about five minutes later.

In a third experiment, twenty ducks 18 days of age were inoculated and ten were put immediately into the chamber with a 50 per cent concentration of oxygen and kept there until the fifth day, at which time the concentration of oxygen was increased to approximately 80 to 85 per cent. The experiment was discontinued on the afternoon of the seventh day. At this time five ducks were dead from the group of ten inoculated with malaria and put in the oxygen chamber, while only one was dead from the group of ten kept as controls in the duck room. The average degrees of parasitemia per 500 erythrocytes in these two groups of ducks are given in Table I.

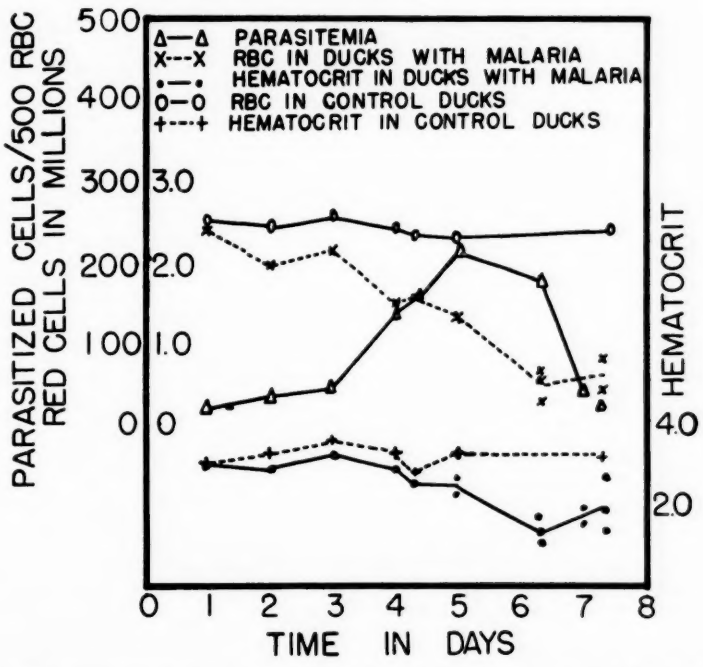


Fig. 4.—The experiment as shown in Fig. 3 was repeated with the ducks kept in the room rather than being put into the oxygen chamber. The parasitemia is much less in this group of birds than in those kept in the oxygen chamber. The anemia and the hematocrit readings are similar to those shown in Fig. 3.

The degree of parasitemia is much greater in the group kept in the oxygen chamber than it is in the control group. The peak of parasitemia apparently was reached at approximately the same time in the two groups. It is not surprising to find a larger number of birds dying in the oxygen-treated group than in the control group, since the parasitemia was much higher in the oxygen-treated group of ducks.

The red blood cell count, the hematocrit readings, and the degree of parasitemia were followed in two groups of ducks, one of which was kept within the oxygen chamber for the entire experiment and the second in batteries in the duck room. A total of forty-four ducks 16 days of age were used. The previously mentioned observations were made upon a different bird each day after which it was discarded. The concentration of oxygen within the chamber, the red blood cell count, the hematocrit reading, and the degree of parasitemia are shown in Fig. 3. The results of similar observations on the control group are shown in Fig. 4. The peak of parasitemia was reached on the fifth day in both groups of ducks; however, the parasitemia was much higher in those kept in the oxygen chamber than it was in the control group. A progressive decrease occurred in the number of red cells and in the hematocrit readings in all ducks regardless of whether they were in the control group or in the oxygen chamber. The red blood cell counts and the hematocrit readings of nonmalarial birds kept in the oxygen chamber with the infected birds decreased during the first four days following which time they rapidly increased to within the range of normal (Fig. 3). Normal ducks used for the controls did not show any significant variation in the red cell count and hematocrit reading during a corresponding interval as shown in Fig. 4.

CLINICAL EFFECT OF OXYGEN ON DUCKS WITH MALARIA

Normal ducks kept in the chamber in the presence of 50 to 60 per cent concentration of oxygen showed no clinical symptoms different from those of normal birds kept in the batteries in the duck room. Ducks inoculated with *P. lophurae* and put immediately in the oxygen chamber progressively became pale and weak and ultimately succumbed in a manner identical with that of the ducks with malaria kept in the duck room. However, ducks with a high degree of parasitemia, an anemia, weakness, and a rapid respiratory rate, when put into the chamber with a 50 to 60 per cent concentration of oxygen, immediately showed an increase in activity, and the respirations decreased in frequency and apparently became deeper. The ducks looked markedly improved almost immediately.

There is little difference in the time that death occurs in groups of ducks put into the oxygen chamber immediately following inoculation when compared with the controls kept in the batteries in the duck room. There is, however, a definite increase in the length of survival of malaria-infected birds put into the oxygen chamber on the fourth day of the disease as compared with a similar group kept only in the duck room as shown in Fig. 5.

In this experiment, twenty-five ducks 16 days of age were inoculated with malaria. On the fourth day at 10:00 A.M. the average parasitemia in ten of these ducks was 298 parasitized cells per 500 red blood cells, and in the afternoon it was 304. Ten of the group of twenty-five ducks were put into the oxygen chamber at 4:00 P.M. on the fourth day of the disease. These ducks were carefully watched during the following forty-eight hours to determine the time of death. The oxygen chamber was kept closed during this time. The

time of death and the concentration of oxygen within the chamber are shown in Fig. 5. At 11:30 A.M. on the sixth day, eight of the ten ducks in the oxygen chamber were dead, while twelve of the 15 birds with malaria kept outside of the chamber were dead. Thirty-five minutes following the time of removal of the two ducks with malaria from the oxygen chamber, one died; the second survived for twenty-four hours after removal. It is obvious from Fig. 5 that ducks with a high degree of parasitemia survive for a much longer time when put into a chamber with a high concentration of oxygen than similarly infected ducks kept at normal atmospheric pressure.

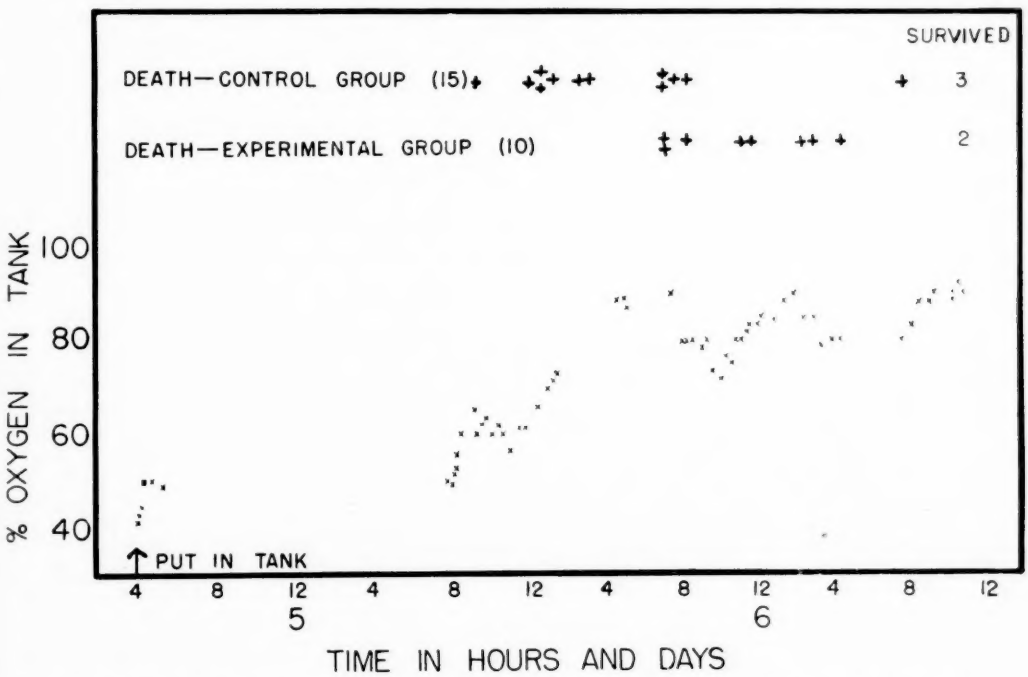


Fig. 5.—The time at which death occurred in the oxygen-treated birds and the controls is indicated here. The concentration of oxygen in the chamber is shown in this figure.

DISCUSSION

The experimental observations as made in these studies indicate that oxygen is beneficial to ducks with a severe malarial infection. The increased amount of oxygen apparently helps to compensate for the decrease in the amount of hemoglobin which is present in the highly infected birds. The anemia results from the rapid destruction of red cells by the plasmodia. In *P. falciparum* infections in man the total decrease in the number of red cells may not be so great in some cases; however, the important factor in all types of acute malaria seems to be the rapidity in which the anemia may develop. The fact that ducks with a severe malarial infection show immediate improvement, either following a transfusion with duck red cells[7] or when they are put in the presence of an increased concentration of oxygen, would suggest that clinical sub-

jects with acute malaria and symptoms of shock should be given a transfusion and placed in an oxygen chamber at the same time that a plasmodial drug is given.

The fact that ducks with a *P. lophurae* infection show a higher degree of parasitemia when kept for the entire period of the disease in the presence of an increased concentration of oxygen does not contradict the beneficial effects that may result from the use of oxygen, since it has been shown experimentally that ducks with a severe infection when placed in the oxygen chamber show immediate clinical improvement. An interval of a few hours is important in the treatment of acute malaria, since it requires such a time for the effects of the plasmodial drugs to manifest themselves upon the parasites, and during this interval the patient may need supportive therapy for the anoxemia. It of course would be useless to kill the plasmodia and permit the patient to die from medical shock. As far as it is known there are no contradictions to the use of transfusions and oxygen in the treatment of a patient with malaria.

Cullen and Boothby and their associates[8, 9] have given the indications and rationale of oxygen therapy. Cullen and associates have emphasized the fact "that there is a 5½ to 7½ per cent increase in metabolism for every degree (Fahrenheit) of elevation in temperature; it would seem logical to provide these individuals with oxygen in excess of that present in the atmosphere."[8] Patients with acute malaria, therefore, would be benefited by oxygen if they had only an increase in their metabolic rate as a result of the fever. Alkalosis present during fever furthermore effects a significant reduction in the arterial oxygen tension even in the presence of a normal arterial oxygen saturation.[8] "The administration of oxygen benefits the patient chiefly by physical methods. That is, an increase in the alveolar concentration increases the partial pressure, thereby increasing the saturation of the blood with a consequent increase in tension in spite of the elevated pH. The break in the vicious cycle of low tension, high pH, and tissue hypoxia may also be effected by the increased amount of oxygen in physical solution in the plasma, which oxygen is readily available to the tissue and not dependent on dissociation of hemoglobin."[8]

Experimental and clinical studies indicate that shock may occur in severe malarial infections.[1, 2, 6] In the treatment of shock from any cause, all efforts should be directed to improve the circulation of blood through the tissue so that oxygen can be delivered at a higher partial pressure. Administration of 100 per cent oxygen is the most direct and specific method to increase the oxygen pressure in the capillaries and tissue fluids.[9] A 100 per cent concentration of oxygen has been administered to patients for forty-eight hours without any evidence of pulmonary irritation.[9]

There is nothing in these experimental studies to indicate that oxygen per se will cure malaria. In fact, these studies show that the parasitemia in *P. lophurae* infection in ducks kept in the presence of a high concentration of oxygen is higher than that which occurs in ducks kept in the presence of the concentration of oxygen as occurs in the normal atmosphere. Experimental studies have indicated that the sudden decrease in the degree of parasitemia which occurs following the peak of parasitemia in *P. lophurae* infec-

tion in ducks may be the result of a lack of oxygen which apparently is essential for the survival and multiplication of these plasmodia in vivo.[10]

The clinical improvement, as observed in ducks with a severe malarial infection when given oxygen, supports the opinion that anoxemia is a significant factor in the mechanism of death in malaria. All of our physiologic and pathologic studies have shown the effects of a lack of oxygen in malaria,[1-4, 11] and now the results from the use of oxygen and the results following transfusions show the therapeutic effects which may be accomplished in the treatment of acute malaria as suggested from these studies in the duck.

SUMMARY

Ducks with a severe malarial infection when placed in an oxygen chamber immediately show marked clinical improvement. They survive for ten to eighteen hours longer than similarly infected ducks kept in the duck room. This improvement is attributed to the greater amount of oxygen available for transmission to the anoxemic tissues.

The use of oxygen in the treatment of acute malarial infections in man is discussed.

REFERENCES

1. Rigdon, R. H.: A Consideration of the Mechanism of Death in Acute Plasmodium Falciparum Infection; Report of a Case, Am. J. Hyg. **36**: 269, 1942.
2. Rigdon, R. H., and Stratman-Thomas, W. K.: A Study of the Pathological Lesions in P. knowlsi Infection in M. Rhesus Monkeys, Am. J. Trop. Med. **22**: 329, 1942.
3. Rigdon, R. H.: A Pathological Study of the Acute Lesions Produced by Plasmodium lophurae in Young White Pekin Ducks, Am. J. Trop. Med. **24**: 371, 1944.
4. Rostorfer, H. H., and Rigdon, R. H.: Anoxemia in Malaria; an Experimental Study in Ducks, J. LAB. & CLIN. MED. **30**: 860, 1945.
5. Kean, B. H., and Taylor, C. E.: Medical Shock in the Pathogenesis of Algid Malaria, Am. J. Trop. Med. **26**: 209, 1946.
6. Kean, B. H., and Smith, J. A.: Death Due to Estivo-Autumnal Malaria; a Résumé of 100 Autopsy Cases 1925-1944, Am. J. Trop. Med. **24**: 317, 1944.
7. Rigdon, R. H., and Varnadoe, N. B.: Transfusions of Red Cells in Malaria; an Experimental Study in Ducks, Am. J. Trop. Med. **25**: 409, 1945.
8. Cullen, S. C., Weir, E. F., and Cook, E.: The Rationale of Oxygen Therapy During Fever Therapy, Anesthesiology **3**: 123, 1942.
9. Boothby, W. M., Mayo, C. W., and Lovelace, W. R.: One Hundred Per Cent Oxygen; Indications for Its Use and Methods of Its Administration, J. A. M. A. **113**: 477, 1939.
10. Rigdon, R. H.: Consideration of the Mechanism by Which a Rapid Diminution Occurs in the Parasitemia in P. lophurae Infection in Ducks. To be published.
11. Rigdon, R. H., and Rostorfer, H. H.: Blood Oxygen in Ducks With Malaria, Nat. J. Malaria. To be published.

DETERMINATION OF NORMAL MEAN CORPUSCULAR WEIGHT (ERYTHROCYTE)

CHEN-TING CH'IN, M.B., CH.B.
NEW YORK, N. Y.

OUR knowledge in various types of anemia has been put on a much more sound basis since the discovery of methods of determining and interpreting the mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration of red cells. It occurred to me that additional help might be derived from the estimation of the mean corpuscular weight of the erythrocyte. This determination was carried out in eighty-one normal subjects including ten Chinese and seventy-one Filipinos. The determination was performed according to the same principles used for the mean corpuscular volume, employing the Wintrobe tube.

METHOD AND RESULTS

All the subjects studied were medical students and doctors of normal development and nutritional status. The erythrocyte count, hemoglobin, leucocyte count, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were found to be within normal limits in each instance. The determination of mean corpuscular weight was made from the same sample of blood.

The capacity of several standard Wintrobe tubes was accurately determined by weighing, on an analytic balance, tubes emptied and filled with distilled water. The capacity of each tube used in these measurements was known to the nearest tenth of a milligram. For simplicity in presentation of the data, a uniform capacity of 0.7 c.c. between the 0 and 100 mm. marks is assumed; in the actual calculations the capacity of each individual tube was used. The technique of the determination was as follows.

The weight of a standard Wintrobe tube was carefully measured. The tube was filled with blood exactly to the 100 mm. mark and centrifuged for at least thirty minutes at 3,000 revolutions per minute. After the hematocrit reading had been taken, the supernatant plasma was removed by capillary pipette without disturbing the cellular sediment. In doing so a column of plasma, platelets, and leucocytes, a few millimeters in length, was left on top of the red blood cells. The tube containing the packed red blood cells and a small amount of plasma was then weighed. When the weight of the empty tube was subtracted from this weight, the weight of the cells and the residual plasma was obtained. The weight of 1 c.c. of plasma made from the same blood sample was measured with a separate set of glassware. From the latter the weight of 0.7 c.c. of plasma was calculated. Dividing the weight of the 0.7 c.c. of plasma by 100, we obtained the weight of a 1 mm. column of the plasma in the Wintrobe tube. By assuming that the density of leucocytes and platelets is equal to that of plasma, and by application of a suitable coefficient to the measured length of the plasma remaining in the tube, we could determine

From the Babies Hospital.
Received for publication, Oct. 28, 1946.

the weight of the residual material. The weight of the blood cells in 1 c.c. of whole blood was derived as follows.

If it is assumed that the tube is of uniform bore, then:

$$\text{Weight of blood cells in 1 c.c. of whole blood in mg.} = \frac{\text{Weight of Wintrobe tube containing the packed cells and residual plasma} - \text{Weight of tube and weight of residual plasma}}{0.7}$$

$$\text{Weight of cells of 1 c.mm. in mg.} = \frac{\text{Weight of blood cells/c.c.}}{1{,}000}$$

$$\text{Weight of cells of 1 c.m. in } \mu\mu\text{Gm.} = \frac{\text{Weight of cells/cc.}}{1{,}000} \times 1{,}000{,}000{,}000$$

$$\text{M.C.W.}^* = \frac{\frac{\text{Weight of cells/c.c.}}{1{,}000} \times 1{,}000{,}000{,}000}{\text{R.B.C./c.mm. in million} \times 1{,}000{,}000}$$

$$\text{M.C.W.} = \frac{\text{Weight of cells/c.c. in mg.}}{\text{R.B.C./c.mm. in million}} \text{ micromicrograms}$$

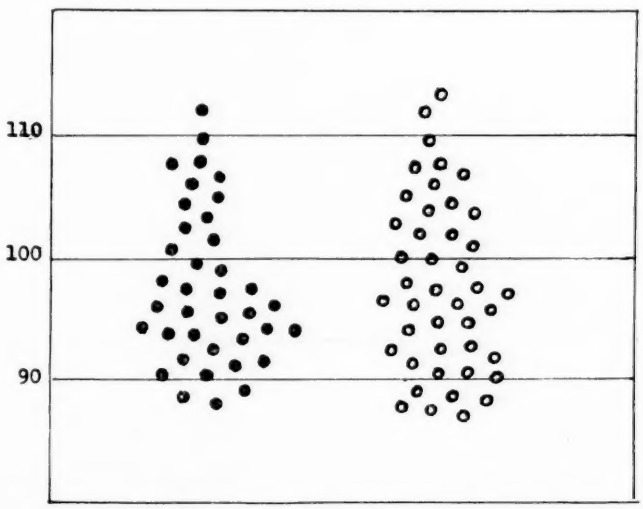


Fig. 1.—Distribution of mean corpuscular weight of thirty-eight men, ●, and forty-three women, ○ ($\mu\mu$Gm.).

The mean corpuscular weights of the eighty-one subjects are presented in Fig. 1. It is significant that in thirty-four of the thirty-eight men and in thirty-five of the forty-three women, the mean corpuscular weight falls within the range of 90 to 110 micromicrograms. The figures for the entire series calculated according to standard statistical methods are shown in Table I. The general hematologic data of the series are shown in Table II.

TABLE I. MEAN CORPUSCULAR WEIGHT ($\mu\mu$GM.) IN EIGHTY-ONE NORMAL SUBJECTS

	NUMBER OF CASES	MEAN $\pm \frac{\sigma}{\sqrt{N}}$	S. D. $\pm \frac{\sigma}{\sqrt{2N}}$
Men	38	96.9 ± 1.04	6.43 ± 0.74
Women	43	96.6 ± 1.29	7.47 ± 0.81
Whole series	81	96.8 ± 0.78	7.02 ± 0.55

*M. C. W., mean corpuscular weight.

TABLE II. HEMATOLOGIC DATA OF EIGHTY-ONE NORMAL SUBJECTS (TEN CHINESE AND SEVENTY-ONE FILIPINOS)

	PRESENT SERIES		FILIPINO STANDARD		AMERICAN STANDARD	
	MEN (38 CASES)	WOMEN (43 CASES)	MEN	WOMEN	MEN	WOMEN
R.B.C. (million)	5.26 (4.24-5.91)	4.74 (4.19-5.35)	5.1 (4.2-5.5)	4.5 (4.0-5.0)	(4.8-6.5)	(4.3-5.5)
W.B.C.	8,000 (5,600-10,500)	7,600 (5,600-9,500)	7,600 (6,300-8,800)	7,500 (6,700-8,500)	(5,000-10,000)	(5,000-10,000)
Hemoglobin (Gm.)	14.2 (11.8-15.8)	13.3 (11.8-14.7	14 (12-17)	12 (11-14)	(14.5-18.5)	(12.5-15.0)
Hematocrit	46 (40-50)	41 (37-46)	(40-50)	(37-45)	(40-50)	(37-45)
Mean corpuscular volume	88.2 (76-95)	87.9 (78-96)	84 (76-95)	83 (75-95)	(75-95)	
Mean corpuscular hemoglobin	27.0 (26-29)	27.4 (26-30)	27 (22-34)		(26.5-31.5)	
Mean corpuscular hemoglobin concentration	30.5 (28-34)	31.4 (29-35)	27 (23-32)		(33-39)	
Mean corpuscular weight	96.9	96.6				

DISCUSSION

The author has no knowledge that the mean corpuscular weight of erythrocytes has been studied previously. Without clinical application in pathologic conditions it is difficult to evaluate the significance of this determination. However, it is reasonable to assume that the mean corpuscular weight should be increased (hyperponderal) in macrocytic hyperchromic anemia and should be reduced (hypoponderal) in microcytic hypochromic anemia. In such conditions the determination of the mean corpuscular weight might serve as a collaborative method in differential diagnosis.

With known methods of study changes of the erythrocyte, other than changes of size, shape, and hemoglobin content, cannot be determined. In this respect the mean corpuscular weight might give additional information, not only in the study of anemia but also in other conditions causing disturbance of fluid and salt balance. However, this is something for future study to prove.

SUMMARY

1. A method of determining the mean corpuscular weight of erythrocytes is presented.

2. The mean corpuscular weight was found to be 96.9 $\mu\mu$Gm. (S. D. = 6.43) for thirty-eight normal men and 96.6 $\mu\mu$Gm. (S. D. = 7.74) for forty-three women, with an average of 96.8 $\mu\mu$Gm. (S. D. = 7.02) for both sexes.

3. The application and significance of this determination are to be ascertained by future study in pathologic conditions.

This study was made in the laboratories of the Cancer Institute of the University of the Philippines, Manila, when the author was stranded there by the Pacific War in 1944. The author wishes to express her gratitude to the members of the laboratories for this privilege and also to Dr. Eugene Stransky of the Philippine General Hospital.

REFERENCES

1. Wintrobe, M. M., and Miller, M. W.: The Volume and Hemoglobin Content of the Red Blood Corpuscle, Am. J. M. Sc. **177**: 513, 1929.
2. Wintrobe, M. M.: A Simple and Accurate Hematocrit, J. LAB. & CLIN. MED. **15**: 287, 1929.
3. Heller, V. G., and Paul, H.: Changes in Cell Volume Produced by Varying Concentrations of Different Anticoagulants, J. LAB. & CLIN. MED. **19**: 777, 1934.
4. Price-Jones, C., Vaughan, J. M., and Goddard, H. M.: Hematological Standards of Healthy Persons, J. Path. & Bact. **40**: 503, 1935.
5. Walters, O. S.: Normal Erythrocyte, Hemoglobin and Packed Cell Volume Standards in Young Men, J. LAB. & CLIN. MED. **19**: 815, 1934.

THE BONE MARROW OF NORMAL CATS

ARTHUR SAWITSKY, M.D.,* AND LEO M. MEYER, M.D.
NEW YORK, N. Y.

INTRODUCTION

SINCE Arinkin's[1] original description of sternal marrow aspiration in human beings, the method has been applied to dogs by Alexandrov.[2] Meyer and Bloom[3] studied normal canine bone marrow obtained by aspiration of the iliac crest, utilizing the technique of Ho and associates[4] who were concerned with investigations in leishmaniasis. Lawrence and co-workers[5] refer to the normal bone marrow of the cat in their paper on feline agranulocytosis. These investigators studied histopathologic sections of bones which were fixed in Zenker's fluid, embedded, and cut. They also examined films made from marrow taken from the humerus and femur which were macerated in human serum and stained with Wright's fluid. This method permitted little opportunity for repeated examinations, and the maximal number of observations made on any animal was three. The majority of animals were sacrificed before they were studied. The procedure to be introduced overcomes this disadvantage because of its simplicity of performance and the availability of the same animal for innumerable punctures. In presenting the data we also compare our observations on the peripheral blood with those of other investigators.

MATERIAL AND METHODS

Studies were made on fifteen apparently normal laboratory cats, both male and female, of unknown age and ranging in weight from 1.69 to 3.43 kilograms each. In the cat the innominate bones articulate with the sacrum and extend caudoventrally, turning medially to unite in the midline to form the symphysis. The ilium extends from the sacrum to the acetabulum. It is contracted in its mid portion and broader at the periphery. The proximal end is thickened, forming the crest, and runs roughly parallel to the vertebral column.

The technique for marrow puncture is as follows: The animal is strapped to the table with dorsum presenting and extremities extended. The hair over the sacral area is clipped and the skin sterilized with any suitable antiseptic. Local anesthesia is not required. The anterior-superior border of the iliac crest is outlined with the fingers of one hand. A sterile 18 to 19 gauge spinal tap needle with straight stylet, about 1½ inches long, is inserted through skin and muscle close to the iliac crest. Upon reaching the periosteum the needle is simultaneously rotated and pushed with a dextro-levo boring movement until the medullary cavity is entered. The marrow cavity has been entered when the needle seems firmly imbedded in the bone and only rarely is a sudden "give" experienced. The stylet is removed, a dry 20 c.c. syringe is attached to the needle, and about 0.1 or 0.2 c.c. of marrow is aspirated. The needle, together with the attached syringe, is then removed from the animal and the drop expelled on a clean glass slide. From the drop white cell and megakaryocyte counts are made with the same technique as for counting peripheral blood. Thin films are made

From the Department of Therapeutics, New York University Medical College.
Received for publication, Nov. 12, 1946.
*Fellow in Hematology.

on several clean glass slides and stained with Wright's stain. Five hundred nucleated cells are counted and the percentages estimated for the differential count from the stained films, utilizing the classification of Vogel and associates.[6] In Fig. 1 is shown the aspiration needle in the iliac crest.

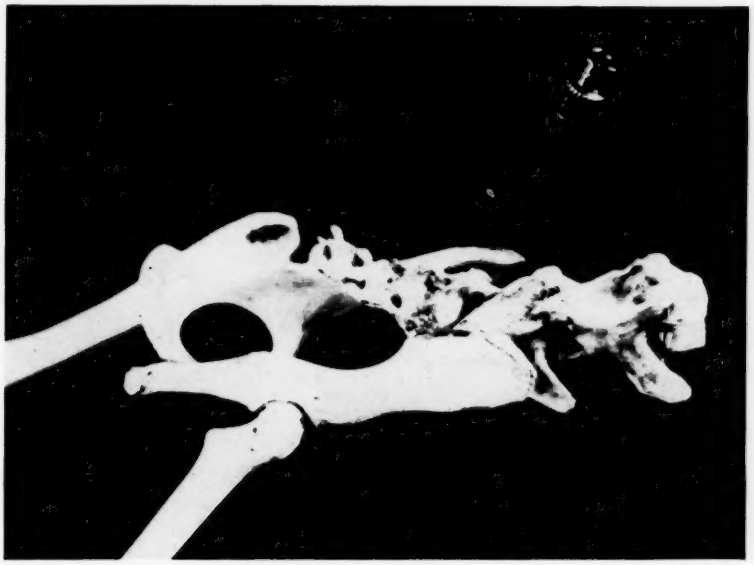


Fig. 1.—Aspirating needle in position in the iliac crest.

OBSERVATIONS

A comparison of the data obtained in the peripheral blood (Table I) with those of other observers (Table II) shows a similarity of results which indicates that the animals utilized in the present study were apparently normal. The only exception is Cat 672 in which the leucocyte count was 40,500 per cubic millimeter. The animal appeared to be well.

The bone marrow from the iliac crest (Table III) shows an increased number of nucleated cells and megakaryocytes as compared with that of human beings and dogs (Table V). The myeloid series constitutes 69.9 per cent of the nucleated cells which are distributed mainly among the segmented (22.5 per cent) and nonsegmented (30.5 per cent) neutrophiles. The metamyelocytes constitute only about 13 per cent of the total number. This increased cellularity of the bone marrow with maturation of cells of the myeloid series is apparently specific for the cat, as it is not observed in either the dog or man. The erythroid and lymphatic cells constitute 20.1 and 9.0 per cent, respectively, which are about the same percentages as seen in canine and human bone marrow. As was noted in dogs, many of the cells of the myeloid series contain no granules, but identification of the cells is not difficult because of the ease in identifying nuclear structures. A comparison of the bone marrow data with that of Lawrence and co-workers (Table IV) is unsatisfactory since these workers included in their tabulation 26.3 per cent degenerated cells. Their erythroid and lymphoid percentages, however, compare favorably with those obtained by us.

TABLE I. PERIPHERAL BLOOD OF NORMAL CATS

CAT	R.B.C.	HEMOGLOBIN	W.B.C.	BANDS	SEGMENTED NEUTROPHILES	LYMPHOCYTES	MONOCYTES	EOSINOPHILES	
1	10.5	6.75	15,600	2.0	56.0	39.0	0.0	3.0	1.0
669	12.2	7.9	10,000	2.5	40.0	44.0	4.0	9.5	--
670	12.5	7.20	14,150	2.0	72.5	17.5	3.5	5.5	--
671	10.5	7.95	21,300	0.5	68.0	24.5	2.5	4.5	--
672	11.0	6.0	40,500	2.0	65.0	23.5	5.0	4.5	--
673	12.5	6.9	21,300	3.0	74.5	18.0	3.0	1.5	1.0
674	13.8	7.3	16,550	0.5	55.0	39.5	1.5	3.5	--
675	12.0	6.6	19,250	1.0	70.0	22.0	2.0	5.0	0.5
676	12.0	9.0	25,300	1.0	70.0	24.75	1.5	2.75	--
677	11.5	7.85	22,800	2.5	68.0	25.0	1.0	3.5	--
678	14.8	8.25	14,400	1.0	65.0	27.5	1.5	4.5	0.5
679	13.8	7.3	20,000	1.0	73.5	15.5	3.0	7.0	--
680	12.0	6.5	18,400	1.5	81.5	13.5	1.5	2.0	--
681	9.5	6.8	28,500	3.0	72.0	18.5	2.5	4.0	0.5
682	10.8	7.05	16,400	2.0	69.5	22.0	2.0	4.5	3.5
Average	11.96	7.22	20,300	1.7	66.7	24.9	2.3	4.4	0.5
Range	9.5 to 14.8	6.0 to 9.0	10,000 to 40,500	0.5 to 3.0	40 to 81.5	13.5 to 44.0	0.0 to 5.0	1.5 to 9.5	0.0 to 1.0

TABLE II. COMPARISON OF DATA ON BLOOD VALUES OF CATS BY DIFFERENT AUTHORS?

AUTHOR	R.B.C.	HEMOGLOBIN	LEUCOCYTES (1,000)	DIFFERENTIAL LEUCOCYTE COUNT (PER CENT)				
				P.M.N.	EOSINOPHILES	BASOPHILES	LYMPHOCYTES	MONOCYTES
Bethe	8.71	--	7.20 to 15.15	--	--	--	--	--
Busch and von Bergen	6.61	--	7.22 to 19.00	54.1	6.5	0	34.5	4.9
Hübner	9.7 to 11.1	12.5 to 15.6	5.15 to 15.20	34.0 to 89.8	1.7 to 4.2	0.1 to 0.4	20.0 to 35.0	2.5 to 3.5
Arndt	7.97	--	--	59.4	5.0	0	33.5	2.1
Kleinberger and Carl	7.74	15.1	10.40 to 29.00	68.5	5.0	0	25.0	1.5
Drastich	8.57	11.3	--	--	--	--	--	--
Scarborough	7.10	10.4	13.00 to 14.00	57.0	5.0	0	32.0	6.0
Wirth	8.00	10.4	10.00 to 15.00	60.0	4.0	0.1	33.4	2.5
Vaulont	7.85	12.8	9.22 to 24.05	41.6 to 84.4	0.8 to 18.0	1	8.4 to 45.2	1.2 to 3.4
Dandsberg	7.23	10.49	8.30 to 35.25	59.3	6.9	0	33.0	0.8
Present authors	7.22	11.96	20.3	68.4	4.4	0	24.9	2.3

TABLE III. BONE MARROW OF NORMAL CATS

CAT	TOTAL MARROW COUNT ×1,000	TOTAL MEGAKARYOCYTES PER CHAMBER	MYELOBLASTS	MYELOCYTES	METAMYELOCYTES	BANDS	SEGMENTED NEUTROPHILES	EOSINOPHILIC MYELOCYTES	SEGMENTED EOSINOPHILES	LYMPHOCYTES	PLASMA CELLS	RETICULOENDOTHELIAL CELLS	MEGALOBLASTS	ERYTHROBLASTS	NORMOBLASTS
1	128	--	0.5	6.0	3.25	26.25	22.5		1.0	13.5	0.0		2.0	2.0	25.0
669	222	13	1.25	5.0	7.0	21.0	20.25	2.0	1.5	5.0	1.0		0.25	4.0	31.75
670	213	22	0.75	6.75	6.0	30.75	25.0	1.0	0.25	6.5	0.5			1.25	21.25
671	204	39	1.0	6.75	8.75	23.75	26.25	1.0	3.0	7.25	1.0		0.5	1.75	18.75
672	230	7	0.25	4.75	9.0	35.25	34.5	1.0	2.25	6.25	1.5			0.75	4.50
673	282	27	1.0	6.6	12.4	38.8	18.8	0.8	1.0	4.8	0.6		0.2	0.2	14.6
674	200	17	1.2	1.6	4.4	30.0	12.8	1.0	3.2	15.8	1.2		1.2	2.0	25.6
675	147	22	0.6	2.4	4.0	19.6	28.4	1.6	2.6	11.6	0.0	0.2		0.4	28.6
676	282	50	0.6	4.8	4.0	33.0	28.8	2.0	0.8	11.6	1.6		0.2	0.2	12.4
677	290	10	1.0	8.4	14.6	38.8	10.6	0.6	0.8	8.4	0.2		0.8	1.2	14.6
678	173	13	2.0	6.0	18.2	40.4	14.4	0.4	1.0	9.6	1.4		0.4	0.0	5.6
679	300	40	0.4	4.6	6.4	30.6	22.0	2.6	3.2	11.0	0.2		0.2	0.8	18.0
680	134	4	0.5	1.5	4.0	32.4	32.1	1.0	1.5	10.7	1.0		0.5	0.5	14.3
681	193	10	0.8	7.4	9.6	42.8	18.4	1.0	2.8	5.0	0.8	0.2	0.8	0.0	10.4
682	151	6	1.0	5.75	7.75	15.5	23.5	0.5	0.75	8.75	0.25		0.25	3.5	32.5
Average	209.9	20	0.82	5.22	7.96	30.59	22.52	1.1	1.71	0.05	0.75		0.35	1.24	18.52

TABLE IV. COMPOSITION OF DATA ON BONE MARROW OF CATS BY DIFFERENT OBSERVERS

BONE MARROW	LAWRENCE AND CO-WORKERS[5]	PRESENT AUTHORS
Number of animals	13	15
Total count		209,900/c.mm.
Megakaryocytes		20/c.mm.
Myeloblasts	1.3	0.82
Promyelocytes	7.6	
Myelocytes		
Neutrophilic	4.6	5.22
Eosinophilic		1.1
Metamyelocytes	9.8	7.96
Nonsegmented		
Neutrophiles	13.4	30.59
Segmented		
Neutrophiles	5.3	22.52
Eosinophiles	1.5	1.71
Lymphocytes	7.8	9.05
Plasma cells	0.3	0.75
Reticulum cells		0.02
Megaloblasts	0.7	0.35
Erythroblasts	6.3	1.24
Normoblasts	8.3	18.52
Unclassified	6.2	
Disintegrated	26.3	
Erythroid-myeloid ratio	0.35	0.29

TABLE V. COMPARISON OF BONE MARROW IN MAN, DOGS, AND CATS

BONE MARROW	MAN[6]	DOG[3]	CAT
Count × 1,000	118.75	144	209.9
Megakaryocytes		41.2	20
Myeloblasts	1.6	0.58	0.82
Myelocytes, agranulocytic	0.1		
Myelocytes			
Neutrophilic	21.5	3.76	5.22
Eosinophilic	0.77	0.26	1.1
Metamyelocytes			7.96
Nonsegmented			
Neutrophiles	30.2	23.50	30.59
Eosinophiles	0.39	0.12	
Segmented			
Neutrophiles	34.0	18.50	22.52
Eosinophiles	0.94	1.56	1.71
Basophiles	0.07	0.02	
Heterophiles		0.02	
Lymphocytes	8.6	9.80	9.05
Pathologic lymphocytes		0.04	
Monocytes		1.20	
Monoblasts		0.14	
Plasma cells		0.82	0.75
Reticulum cells	0.25	0.54	0.02
Hematogones	3.1	0.44	
Megakaryocytes	0.2		
Megaloblasts	0.14	1.02	0.35
Erythroblasts	7.1	2.50	1.24
Normoblasts	22.6	53.18	18.52

SUMMARY

1. A method is presented describing the technique for obtaining bone marrow from the iliac crest of the cat by aspiration.

2. Data are given showing the cellular constituents in the peripheral blood and bone marrow of normal cats.

3. A comparison of the bone marrow findings in cats with those of dogs and man is made, utilizing the method of aspiration in all three groups.

REFERENCES

1. Arinkin, M.: Methodology of Examining Bone Marrow in Live Patients, With Hematopoietic Diseases, Vestnik Khir. (No. 30) **10**: 57, 1927.
2. Alexandrov, A. P.: Die Morphologie des Sternumpunctates von Hundem, Folia haemat. **41**: 428, 1928.
3. Meyer, L. M., and Bloom, F.: Bone Marrow of Normal Dogs, Am. J. M. Sc. **206**: 637, 1943.
4. Ho, E. A., Chu, H. S., and Yuan, I. C.: Ilium Puncture; A Simple Method for Obtaining Bone Marrow From Dog, Chinese M. J. **58**: 679, 1940.
5. Lawrence, J. S., Syverton, J. T., Shaw, J. S., and Smith, F. P.: Infectious Feline Agranulocytosis, Am. J. Path. **16**: 333, 1940.
6. Vogel, P., Erf, L. A., and Rosenthal, N.: Hematological Observations on Bone Marrow Obtained by Sternal Puncture, Am. J. Clin. Path. **7**: 436, 1937.
7. Landsberg, J. W.: The Blood Picture of Normal Cats, Folia haemat. **64**: 169, 1940.

LABORATORY METHODS

REACTIONS OF VARIOUS PARASITIC ORGANISMS IN TISSUES TO THE BAUER, FEULGEN, GRAM, AND GRAM-WEIGERT METHODS

R. D. LILLIE*
BETHESDA, MD.

IN THE course of some experimental comparisons of the Best carmine method and the Bauer[1] reaction for glycogen, some cat muscle containing Sarcosporidia chanced to be included in the test material. With the Bauer method on low magnification the Sarcosporidia appeared red purple in a pale gray blue background. On higher magnification they were seen to contain numerous red purple granules in their cytoplasm around the nucleus in the equatorial area of fusiform parasites. After salivary digestion according to Bensley[2] the Sarcosporidia were still red by the Bauer method, while muscle glycogen no longer stained. With Best's carmine method Sarcosporidia do not take the red stain.

This experience led to more extended examination of various tissue parasites by the Bauer method. In most instances it was necessary to use paraffin sections of old material fixed for other purposes in 10 per cent formalin, Orth's fluid, Helly's fluid, or other fixatives.

METHODS

The technique of the Bauer reaction and the composition of the Schiff reagent used for both the Bauer and Feulgen tests were substantially as recently reported.[3] By this method glycogen is stained a deep purplish red. The reaction may be prevented by salivary[2] or by enzyme digestion.[3] Cartilage matrix is usually colored a deep reddish purple, contrasting fairly well with the lighter purplish red glycogen in the cartilage cells. Cartilage matrix sometimes remains unstained. The staining of cartilage matrix is not affected by prior salivary, diastase, or ptyalin digestion.

Bauer[1] stated that mucus did not react to this method; however, as Bensley[2] notes and as we found, in mammalian material, mucus is usually stained purplish pink to light purplish red. This staining reaction persists after salivary, ptyalin, or diastase digestion. Mucous stains well by this method after aqueous formalin or Orth fixations.

Mast cell granules were not demonstrated by either the Bauer or the Feulgen techniques in human, cat, or rat material in which they were readily demonstrable with our azure-eosin technique.

When enzyme digestion tests were used, the digestion was performed on deparaffinized, hydrated sections without collodion treatment and after digestion sections were dehydrated and collodionized as previously mentioned before testing with the Bauer technique. The technique is as recently reported by us.[3] For comparison the Feulgen, Gram, and Weigert methods were used as well on most of the material.

The Feulgen technique used herein employed the same Schiff reagent as described by us for the Bauer method[3] and followed essentially the procedure outlined by de Tomasi[4] and by Stowell[5]: Deparaffinize and hydrate sections as usual. Rinse for one minute in N/1 HCl

From the Pathology Laboratory, National Institute of Health.
Received for publication, Oct. 18, 1946.
*Medical Director, United States Public Health Service.

and hydrolyze for twenty minutes at 50° C. in N/1 HCl. Rinse for one minute in cold N/1 HCl and immerse for two hours in Schiff reagent. Give three successive ten-minute baths in closed Coplin jars in acid sulfite mixture (10 c.c. of 10 per cent $NaHSO_3$, 10 c.c. of N/1 HCl, and 200 c.c. of distilled water). Wash for five minutes in running water. Counterstain briefly (thirty seconds) in 0.02 per cent alcoholic-fast green FCF, dehydrate in alcohols, clear in xylene, and mount in clarite.

The Gram-acetone-safranin technique was as published in 1928.[6] The Gram-Weigert technique is as follows: Deparaffinize and hydrate as usual. Stain for ten minutes in carbolfuchsin and wash in water. Stain for two minutes in ammonium oxalate crystal violet. Drain, blot, and apply three changes of Weigert's iodine to a time of thirty seconds total. Wash in water. Blot and decolorize with two or three changes of Weigert's aniline xylene (50:50), blotting between changes. Wash in three changes of xylene and mount in clarite.

OBSERVATIONS

Protozoa.—Muscle was resectioned for study from seven monkeys *(Macaca rhesus)*, four more cats *(Felis cattus)*, two Texas armadillos, one cotton rat *(Sigmodon hispidus)*, one mouse *(Macaca musculus)*, one ground squirrel *(Citellus beecheyi)*, and seven wood rats *(Neotoma micropus)*. By the Feulgen method the Sarcosporidia presented small oval red purple nuclei. They were Gram negative and Weigert negative. With the Bauer method there was much finely granular purplish red material about the nuclei of the parasites, so that the affected muscle fibers stood out conspicuously at very low magnifications.

Digestion with saliva, ptyalin, and malt diastase was resisted so that the granules remained Bauer positive in the first cat. Contrariwise, ptyalin and malt diastase digestions completely destroyed the Bauer-positive material in the armadillos, monkeys, wood rats, mouse, cotton rat, and two of the cats, thus identifying it as one of the hexose polysaccharides digestible by those enzymes. In the ground squirrel and the other two cats, most of the colonies lost their Bauer-positive material but some colonies and some individual organisms did not. That this increased resistance to digestion may have been artefact is indicated by the fact that Bauer-positive material remained after diastase digestion only in one of four sections from the ground squirrel tissues.

Further sections from the first cat were subjected to diastase digestion for periods of 30, 60, 90, and 120 minutes. While many Bauer-positive granules remained after 120 minutes, there was a very evident reduction in their number and size, and some cysts contained some or all Bauer-negative parasites. Shorter digestion periods left more and more Bauer-positive material. Altogether it appears that the positive Bauer reaction of these granules in the Sarcosporidia depends on glycogen or a similar polysaccharide.

The brains of nine mice, infected experimentally with toxoplasmosis and fixed in Orth's fluid, were resectioned. In four no parasites were found. In five microcysts containing Toxoplasma were identified. The Feulgen reaction demonstrated numerous tiny oval vesicular red purple nuclei in all five. In three of these the Bauer reaction showed many rather vague purplish red granules in a pale blue matrix. In the other two the parasites were apparently Bauer negative. Gram-acetone and Gram-Weigert stains were done on the three Bauer-positive specimens. Parasites were red and pale pink, respectively, by these methods.

Goodpasture stains were done according to Perrin's technique[7] and in the three brains in which parasites could be found they were stained brown.

After digestion tests with ptyalin and with diastase, Toxoplasma still stained red with the Bauer method. The Bauer-positive material was perhaps more clearly demonstrated as a deep purplish red perinuclear deposit, sometimes masking the nucleus; than in undigested material.

Encephalitozoa were found in six of seven mouse brains resectioned for study. They occurred as oval plump organisms in focal lesions. Some in the Gram stain are longer and slenderer. With this Gram-acetone method they stained pale to fairly deep violet but not blue black. With the Gram-Weigert method the organisms varied from red through purple to violet and sometimes blue black. Feulgen preparations from adjacent sections failed to demonstrate the organisms in the same focal lesions. In Bauer preparations the cells of the focal glioses assumed a vague pink color. Among them there were discerned with difficulty clear, oval refractile bodies of the same size and position as the organisms in Gram-Weigert and Goodpasture preparations. After diastase or ptyalin digestion, however, the organisms were more clearly discerned as oval purplish pink rings with clear centers lying in a light blue background.

The material for study of Encephalitozoon and Toxoplasma was resectioned from animals inoculated with the respective organisms by Perrin.[7]

Examples of endoerythrocytic and exoerythrocytic forms of *Plasmodium gallinaceum* were furnished me by Dr. L. R. Hershberger. In agreement with his usual findings the parasite nuclei stained purplish red by the Feulgen technique. Gram, Weigert, and Bauer reactions were negative.

Sections were recut from the kidneys of twelve mice infested with *Klossiella muris*. In eleven of these more or less numerous parasites were demonstrated. Rosette forms with peripheral budding showed in the buds small oval leptochromatic vesicular nuclei staining light red purple with Feulgen. Clusters of small intratubular round forms were Feulgen negative or contained faintly stained very fine single to multiple granules. In somewhat larger forms Feulgen-positive granules were still lightly stained and usually multiple. In medium-sized round forms dense red purple diplococcoid granules were present dispersed through the cytoplasm. Organisms of this last size were sometimes red purple or less often blue black with the Gram-Weigert technique. Sometimes as many as one-fourth to one-third of the individuals in a given intratubular cluster were so stained, while the rest were Gram negative. With the Gram-acetone method fewer organisms were stained violet or, occasionally, blue black, while other organisms in the same intratubular cluster presented multiple diplococcoid chromatin granules stained red by the safranin counterstain. By the Bauer method most organisms in all animals were Bauer negative. However, in all but one mouse, irregular masses and medium to fine granules of brilliant purplish red material were found in occasional to moderately numerous medium-sized parasites. Liver sections on the same slide in six of eleven mice showed moderate amounts of Bauer-positive glycogen in the liver cells.

Kidney sections from nine of these mice were successfully carried through ptyalin and diastase digestion tests, and the subsequently performed Bauer

reaction revealed in four of them occasional to moderately numerous parasites containing brilliantly purplish red granules. The six livers previously noted had lost all of their glycogen after the diastase digestion and all, or all but traces, after ptyalin.

Trypanosoma equiperdum in methanol-fixed thin blood films is Bauer negative, Weigert negative, and Gram negative. Trophonuclei stain pale purplish red with Feulgen and blepharoplasts were not discerned. However, lymphocyte nuclei are also rather lightly stained. Other fixations might give better results.

Trypanosoma cruzi was demonstrated only in one monkey heart and in two mouse hearts of several resectioned for study. The organisms occurred as the usual Leishmania forms in cysts within muscle fibers. Their cytoplasm stained light green, trophonuclei light red, and blepharoplasts as deep red purple rods in Feulgen-fast green FCF preparations. In Gram-acetone-safranin stains blepharoplasts were deep red, trophonuclei light red, and cytoplasm pink. With the Gram-Weigert technique cytoplasm and trophonuclei were pale pink and blepharoplasts deep violet to blue black. The Bauer reaction was negative, though a moderate amount of glycogen was shown in a liver section on the same slide with the one mouse heart and in muscle fibers of the other mouse heart.

Eight cases of amebiasis were resectioned for study. *Endamoeba histolytica* was readily found in all. The organisms were Gram negative and Weigert negative. The nuclei stained relatively faintly by the Feulgen technique. Many amebae contained more or less copious Bauer-positive granular material in their cytoplasm, but in some organisms the cytoplasm was blue gray and vacuolated and in some contained only a few purplish red granules. After diastase digestion all presented blue gray cytoplasm.

This observation extends that of Meriwether[8] who reported the selective staining of amebae by Best's carmine but did not identify the Best-positive material as glycogen. It would appear from the present observations that *Endamoeba histolytica* often contains glycogen or a similar polysaccharide.

Coccidia *(Eimeria stiedae)* were demonstrated in greater or less profusion in eight of eleven rabbit livers resectioned for study. With the Feulgen method most of the large coarsely granular parasites, whether intraepithelial or free, were Feulgen negative. Some small forms showed red purple granules or green granules with red purple margins. These were not found in five rabbits. In some rabbits there were found, in addition to the typical coccidia, masses of pale green cytoplasm without granules, in which were rings or spirals of small red purple fusiform or bacciliform chromatin granules. With the Gram-acetone technique granules were stained red and shells pink. The Gram-Weigert method gave blue black or deep violet coarse granules in the coccidia, while their shells were stained in varying tones from pink through violet to deep violet or blue black. The Bauer reaction demonstrated a pink to purplish red matrix in the coccidia, in which the refractile or unstained coarse granules were discernible. This material was present also in the coccidia in livers collected six to forty hours post mortem, in which all glycogen had disappeared from the liver cells. Application of Bensley's salivary digestion test to collodionized sections removed the pink matrix in two rabbits, at the same time removing all of the fairly copious

glycogen from the liver cells. In a third rabbit the same test apparently decreased the intensity of the Bauer reaction in the coccidial cytoplasm. Use of the diastase and ptyalin digestion tests on uncollodionized sections also removed Bauer-reacting material from nearly all of the parasites. All glycogen in liver cells was removed by the enzymes.

Bacteria.—Various sections of acute ulcerative appendicitis, of chronic appendicitis, and of animal stomachs and intestines, with or without acute ulcerative lesions, were examined. Various Gram-positive and Gram-negative bacilli and cocci were demonstrated with Gram and Weigert techniques. Gram-positive organisms were more plentiful with the Weigert method. These organisms all failed to stain by the Feulgen and Bauer techniques. However, a few Bauer-positive, Gram-negative branching filamentous molds were seen in a slough in amebic dysentery and in the margin of a cutaneous blastomycotic sinus. Material from five cases of human anthrax was included. The bacilli were usually partly Gram positive and partly negative with the Gram technique. The organisms were Bauer negative. A *Bacillus subtilis* contamination of lung tissue revealed numerous Gram-positive bacilli by the Gram and Weigert methods. The organisms were Feulgen negative and Bauer negative.

A series of four mice inoculated intracerebrally with material under study for the presence of poliomyelitis virus developed empyema of the cerebral ventricles. The pus cells contained a variable small amount of glycogen which was destroyed by the diastase digestion test. In the pus were clumps of small bacilli stained light blue with deep purple (metachromatic) polar granules by the azure eosin stain. With the Weigert stain the bacilli were barred, beaded, bipolar, or solidly blue black. The Bauer method revealed them as slender purplish red rods, which were possibly more distinct after diastase digestion than without it. The Feulgen method failed to stain these diphtheroid bacilli.

Guinea pig spleens and lungs containing numerous tubercle bacilli by the Ziehl-Neelson technique showed no organisms with Feulgen, Bauer, or azure-eosin methods. The bacilli stain blue black by the Weigert and Gram techniques if exposed a sufficient length of time or if heated in the crystal violet solution.

Four chick embryo yolk sacs fixed in Regaud's fluid were resectioned. Azure-eosin stains demonstrated numerous *Rickettsia orientalis* in all. These were not stained in otherwise good Feulgen or Bauer preparations. Tissues of four psittacine birds dead of acute psittacosis were resectioned. Numerous *Rickettsia psittaci* (LCL bodies) were demonstrated with azure eosin; none were demonstrated with the Feulgen method. Fresh sections from four rodents dead of acute tularemia presented numerous organisms in azure-eosin preparations; none presented organisms in Feulgen stains.

Fungi.—Histoplasmosis *(Histoplasma capsulatum)* was studied in the liver and spleen of two dogs and two hamsters, in the lung of a guinea pig, and in the omentum and abdominal wall of a rat. Fixation was with 10 per cent formalin, Helly, or Zenker, all three in one dog. The Feulgen method revealed tiny vesicular light red purple nuclei separated by a clear zone from a pale green capsule. In some of the Zenker-fixed material these nuclei were not demonstrated by Feulgen and stained poorly in comparison with those in formalin-

fixed material from the same dog with the iron-hematoxylin-van Gieson technique. With the Gram-acetone-safranin technique histoplasma nuclei usually stain red to pink; capsules stain paler or much paler. In the rat and in the liver of one hamster a few organisms stained blue black or violet, but most of them were Gram negative. A variable proportion of the organisms were Gram positive when the Gram-Weigert technique was used: none in one animal, few in another, one-third in one, "many" in two, and one-half to two-thirds in one. Usually Gram-positive organisms were stained solidly blue black; however, sometimes only the capsules were stained blue black or violet, while nuclei or nuclei and cytoplasm were stained red by the fuchsin prestain.

Results with the Bauer reaction were striking. Organisms were readily identified as fine red purplish rings surrounding a clear zone about the centrally placed, blue or pale blue nucleus. Organisms were more readily found than with azure-eosin or Giemsa stains. After salivary digestion the histoplasma capsules were still Bauer positive. Ptyalin and diastase digestion tests gave the same results in the five animals so tested.

In another subsequently studied animal numerous Bauer-positive rings without central nuclei were found in the foam-cell granulation tissue where no histoplasma could be found with azure-eosin stains. In other areas where histoplasma was demonstrable with the usual azure-eosin stain, the Bauer-positive rings were present in equal numbers and here contained the central blue stained (hematoxylin) nucleus as previously described. This indicates that the sulfurous acid-fuchsin method may be used to demonstrate non-nucleated and presumably dead histoplasma as well as the nucleated and presumably surviving forms. I am indebted to Dr. L. L. Ashburn for the observations on this dog and for material.

Five animals infected with *Haplosporangium parvum* were studied. The tissue studied was lung and was fixed in 10 per cent formalin in four of the animals and in Orth's fluid in the fifth. The animals comprised one rhesus monkey, one cotton rat, and one white mouse, all infected in the laboratory, and two Perognathus mice captured in the infected state. In the first three all of the organisms were Gram negative; in the last two a few were Gram positive, most Gram negative with the Gram-acetone technique. With the Gram-Weigert technique a few blue black organisms appeared in one of the first three animals, while in the last two most of the fungi were blue black. The Feulgen reaction revealed no Feulgen-positive material in any of the fungi, though host nuclei were well stained. With the Bauer reaction the capsules of the fungi appeared as thick deep purplish red rings, both with and without antecedent salivary digestion.

Subcutaneous masses from three guinea pigs, fixed in formalin, and brains from 3 mice, fixed in Orth's fluid, were studied in torulosis. In all numerous *Torula histolytica* were present. From 10 to 50 per cent of the yeasts were Gram positive with the Gram-acetone method. The Gram-Weigert method showed nearly all blue black in the three guinea pigs. It was not used on the mouse brains. In the guinea pigs the fungi were apparently Feulgen negative, while in the mouse material small round or oval nuclei were found. These

stained light red purple to pale lilac. With the Bauer method the outer shells of the yeasts appeared as deep purplish red to purplish violet rings. These were apparently somewhat redder in sections subjected to preliminary salivary digestion. Small forms appeared as solid red globules.

A Peromyscus mouse and seven white mice experimentally infected with *Coccidioides immitus* were reused for this study. Thirteen blocks from omental and visceral masses, testes, and lungs were used. Fixation was with buffered 10 per cent formalin for the seven white mice and with Orth's fluid for the Peromyscus. The fungi were regularly Feulgen negative except on one trial where pink nuclear staining was obtained, and a deteriorated Schiff reagent may have been used for this. Fungi were red by the Gram-acetone-safranin method except for a few blue black endospores in one and a few violet fungi in two of the thirteen blocks. With the Gram-Weigert technique a variable proportion were blue black or deep violet, from as few as one-fourth to nearly all in the several blocks studied. Endospores and small fungi were more often Gram positive than large forms or empty shells. Regularly the thin shells of the mature fungi and the shells of endospores and small fungi were red to red purple with the Bauer technique, and often the fungi contained solid purplish red masses within them, both endospores and larger forms. The five livers observed were glycogen free.

Application of ptyalin and diastase digestion tests to material from five animals reveals that the organisms still presented purplish red shells and endospores when the Bauer reaction was applied after the digestion, but no internal masses staining red persisted.

In Case A-2333, *Monilia candida* endocarditis, numerous small oval yeasts were demonstrated in the mitral thrombus. The yeast cells stained green with the Feulgen method. Within the cells were indistinct or distinct fine granules or delicate tiny rings of material staining purplish pink to red. The yeast cells were nearly all Gram positive by the Gram technique; all stained blue black by the Weigert method and deep purplish red by the Bauer method. After diastase digestion the positive Bauer reaction was unaltered.

Three cases (S-454, S-1187, and S-9410) of cutaneous blastomycosis and one case (A-888) of blastomycotic pulmonary abscesses were studied. The yeast cells were either Feulgen negative, or questionably pale, or very pale red purple nuclei were present. The yeasts were Gram negative, and, by the Weigert technique, only occasional cells stained violet in one of the four cases. The cell walls were stained deep purplish red by the Bauer method, both with and without preceding diastase digestion. Glycogen in epidermal cells and sweat gland cells was destroyed by the diastase.

Kernohan[9] has previously indicated the presence of a substance stained by Best's carmine method in the capsules of *Histoplasma capsulatum, Coccidiodes immitus, Torula histolytica, Blastomyces hominis,* Mucor, and Monilia. The present study parallels but does not repeat these findings and further identifies the capsular substance as one resisting enzyme digestion with diastase and ptyalin.

A section of guinea pig omentum with experimental mycetoma was studied. The fixation was with 10 per cent formalin. The organism was *Allescheria boydii*. The tissues contained compact rings of closely felted, refractile brownish yellow, tortuous septate mycelia. The enveloping membrane was more often brown, sometimes blue black, with the Gram-Weigert method. It was unstained by the Gram-acetone method. The Bauer method showed most of the filaments in their natural brown. A few of the most distal, ramifying filaments were red, with or without antecedent diastase digestion. No chromatin was demonstrable with the Feulgen method.

Formalin-fixed liver and spleen from a rabbit infected with *Aspergillus fumigatus* presented Gram-negative mycelia with the Gram-acetone technique. With the Gram-Weigert method part of the mycelia were blue black and part red. With the Bauer method a very delicate red to purple capsule was recognized outside a violet protoplasm and crossing at the septa. This Bauer-positive material resisted salivary and diastase digestions.

The mycelia of chromoblastomycosis in the formalin-fixed omentum of a rat were partly blue black, partly red, and partly brown by the Gram-Weigert method. Staining was apparently restricted to an outer capsular substance, so that the mycelia appeared as hollow tubes. With the Gram-acetone and the Bauer techniques the mycelia apparently retained their natural brown color for the most part. Some filaments and, especially, spore-like enlargements presented Bauer-positive walls, particularly after diastase digestion.

In Case S-8442, mycetoma of wound of hand, the granules were Feulgen negative and Gram negative with the Gram-acetone method. By the Gram-Weigert technique there were few to moderately numerous ramifying septate Gram-positive mycelia. These appeared foamy, vacuolated, or barred blue black on a pink background. With the Bauer method the granules were composed of a dense felt work of light purplish red hollow, rather thin-walled tubules, which remained Bauer positive after diastase and ptyalin digestion.

In Case S-14788, Cephalosporium mycetoma, the granules were again Feulgen negative and Gram negative with the Gram-acetone method and again contained a few branching, foamy, vacuolated, or solid blue black mycelia by the Gram-Weigert method. The Bauer reaction again revealed a close feltwork of light red purple tubules, which remained Bauer positive after diastase digestion.

In Case A-2884, Cephalosporium mycetoma, multiple sections of sinus tracts presented numbers of large granules composed of wide dichotomously and laterally branching mycelia with frequent transverse septa and scattered bulbous enlargements to twice ordinary width and about thrice the mycelial width in length. These bulbous enlargements were relatively thick walled. The margin was partly composed of bulbous enlargements similar to the foregoing in structure. The mycelia and bulbs were Feulgen negative and Gram negative. With the Weigert stain part of the mycelia were stained pink, part presented violet walls, and some presented transverse blue black bars. Some violet walled mycelia ramified beyond the hyaline Gram negative-matrix into the surrounding

pus. With the Bauer method the mycelial walls and septa stained deep purplish red, with or without antecedent diastase digestion. The mycelia were not acid fast.

In Case A-3072, Penicillium species abscess of lung, imbedded in the pus of the abscess cavities and in giant cells were fragments and radiating stellate masses of broad septate mycelium with closely spaced septa, scattered bulging thicker-walled segments, and separate oval yeast-like globules similar in short diameter to the bulging segments. Lateral and dichotomous branching were observed. Mycelia were "negative" to the Ziehl Neelson, Feulgen, and Gram methods. Most filaments stained pink; a few stained violet, and occasional ones were solidly or barred blue black by the Weigert method. Bauer stains, both before and after diastase digestion, colored the walls and septa a deep purplish red.

Four cases of actinomycosis (A-478, A-1914, S-12263, and A-2187) and one case of maduramycosis (S-17813) presented more or less numerous granules composed of a central feltwork of narrow mycelial threads and a peripheral hyaline matrix whose outline varied from granule to granule in each case from smooth or lobate to tongue-like narrow prolongations with bulbous tips. The mycelial threads only infrequently presented dichotomous branching. They were Feulgen negative. Many stained blue black, some solidly, some barred or beaded, and some red with the Gram method, and nearly all were blue black by the Weigert method. The hyaline matrix and border was Gram negative by both methods. With the Bauer method some granules presented a vague purplish pink granular deposit centrally; in some this purplish pink material was vaguely filamentous, and in a few more sharply defined purplish red filaments were discerned. After diastase digestion vague and definite filaments were often more readily discerned.

Of the foregoing cases, only the last was studied culturally.

In Case S-22853, mycotic gastric ulcer, the Gram-acetone method revealed a profusion of red filaments and fine and coarse rods in the ulcer base. A minority of these organisms were blue black by the Gram-Weigert method. The filaments and rods were unstained by the Feulgen and Bauer methods.

Sufficient material from one case only of botryomycosis (S-11861) was available for restudy. One to several "granules" were found in suppurating sinuses in each of the five sections. Azure-eosin stains revealed a hyaline eosinophilic border zone, marginally serrate, lobate, or bulbous. Next within this was a zone of densely packed cocci, sometimes poorly stained and sometimes deep blue. The centers were composed of more or less closely packed, deeply basophilic cocci lying in clusters, pairs, or short chains. With the Gram stain the cocci were Gram positive in some granules and Gram negative in others. By the Weigert method there were always considerable numbers of closely and loosely packed Gram-positive cocci occurring in clusters and lying in a hyaline pink matrix. The marginal serrate or club zone was hyaline and stained pink to fairly deep red.

By the Bauer method, with and without antecedent diastase digestion, the granules stood out under low magnification as conspicuous purplish red

masses. The border or club zone stained pale gray or blue gray. The cocci appeared as pale blue bodies. Among the cocci were numerous fine red granules and short and narrow red bacilli, fewer pale pink rods containing red granules, and a few longer red filaments which occasionally showed branching. Bacillary and filamentous structures were best discerned in marginal areas. Central areas presented too much vague red granular material to permit discernment of detail. Occasionally coarser pink filaments with clear centers were discerned, and in cross section these appeared as pink to red rings about the same size as the cocci. Red filaments and bacilli were perhaps 0.5 μ in thickness; cocci were about 1 to 1.5 microns.

The existence of small bacillary and occasionally filamentous Bauer-positive elements in the granules of this case of botryomycosis in addition to the usually described Gram-positive staphylococci is a matter of interest requiring further investigation to decide as to its possible etiologic significance.

Metazoa.—Tissues from eight animals were resectioned for study and Schistosome eggs were demonstrated in seven of them. Two species, *Schistosoma mansoni* and *Schistosoma japonicum*, were included in the material, and the animals included six experimentally infected hamsters and a naturally infected dog. Some adult parasites were found in veins. Nuclei of adult parasites and embryos were red purple by the Feulgen method, just as were the host cell nuclei. With the Gram-acetone technique the shells were usually pink but sometimes pale violet. With the Gram-Weigert method the shells varied from pink to blue black, being perhaps most often violet. Using the Bauer technique the shells were usually deep purplish red. Fragmenting shells stained similarly and the granulomata surrounding fragmenting eggs or replacing them often contained globules and masses of purplish red to purplish pink hyaline material in the cytoplasm of the epithelioid cells. After diastase and ptyalin digestion tests the shells of the ova still stained red purple with the Bauer method, but the cytoplasm of the epithelioid cells became Bauer negative.

The implication that the cells of the granuloma convert the chitin of the Schistosome eggshells into a polysaccharide which is digestible by ptyalin and by diastase, while the chitin itself is not so digestible, appears to warrant further study.

The livers of four wild rodents infected with numerous doubly operculate eggs of *Capillaria (Hepaticola) hepatica* were resectioned for study. The infested animals were a woodchuck (6240), a cottontail rabbit (8385), a jackrabbit (8788), and a squirrel (8789) from Western Montana. These ova, when stained with azure eosin, presented several blue nuclei, coarsely granular pink cytoplasm, a hyaline, refractile inner shell, and a refractile, radially striate outer shell. The operculae appeared as apparently open spaces. The embryo nuclei stained purplish red by the Feulgen method, the refractile inner shell was tinted yellow to orange, and the cytoplasm and the radially striate outer shell stained green. With the Gram stain the refractile inner shell stained pink to violet, the cytoplasm was largely pink, sometimes containing violet granules, and the outer shells were refractile to pale pink. The nuclei stained red. With the Weigert method the refractile inner shell stained deep red, the operculae were filled by

deep violet to blue black plugs, the cytoplasm was violet to pink, and the radially striate outer shell was pink. Diastase and ptyalin digestions did not appreciably alter these reactions.

Inner and outer shells remained unstained or yellowish by the Bauer method, the operculae were filled by deep purplish red plugs, nuclei were blue, and cytoplasm was granular and light purplish red or less often light blue. Diastase digestion rendered the cytoplasm of most of the eggs Bauer negative; ptyalin was less effective but still destroyed most of the Bauer-positive granular material in the cytoplasm. The opercular plugs still stained deep purplish red after digestion with either enzyme.

In a Norway rat (1364), cysticercosis of liver, most of the cyst walls and cestode tissues were Gram negative, Weigert negative, and Bauer negative. In one section, apparently scolex, much granular purplish red material was present in the cestode tissue with the Bauer method, and the Weigert preparation gave violet granules in the same location. This section possessed the most Feulgen-positive nuclei, but fair numbers of these were present also in the cyst walls. After ptyalin and diastase digestion tests the Weigert-positive material was destroyed, and the Bauer-positive material in the scolex persisted after ptyalin but not after diastase digestion.

In a snowshoe rabbit tapeworm (15720), the calcareous globules were deep blue by azure eosin, violet with Weigert, and unstained by the other methods. Nuclei were stained purplish red with the Feulgen technique. The cuticle was deep blue with azure eosin and red with Weigert and Gram techniques. The Bauer preparation showed numerous granules and globules of purplish red material in the stroma. After a ptyalin digestion test this material became Bauer negative. Similarly, Bauer[1] has shown the presence of glycogen in stroma cells and elsewhere in *Fasciola hepatica,* using iodine, Best and Bauer methods, and salivary digestion tests with the two latter stains.

In Case S-4147, appendix containing Trichuris and Ascaris eggs, the eggs appear yellow by the Bauer method, brown to yellow by the Feulgen, pink to red by Gram-acetone, and their shells stained red to purple by Gram Weigert.

In Case S-18128, *Onchocerca volvulus* in skin, the chitin of adults stained pale reddish purple with the Bauer method, whereas the same parasites contained numerous Feulgen-positive nuclei. Feulgen-negative (dead?) parasites gave a denser red purple chitin stain with the Bauer method. The chitin was Feulgen negative, Gram negative, and Gram-Weigert negative.

SUMMARY

Sharply stained nuclei were demonstrated by the Feulgen method in the following: Sarcosporidia, Toxoplasma, *Klossiella muris, Trypanosoma cruzi, Plasmodium gallinaceum;* in eggs, larvae, and adults of *Schistomosoma japonicum* and *Schistomosoma mansoni;* in larvae and adults of tapeworms, roundworms, and *Onchocerca volvulus;* and in eggs and larvae of *Capillaria hepatica.* Poorly stained or dubiously Feulgen-positive nuclei were encountered in Encephalitozoon, *Eimeria stiedae, Endamoeba histolytica, Torula histolytica,*

Blastomyces, and *Histoplasma capsulatum.* The mycelial fungi, bacteria, rickettsiae, *Coccidioides immitus,* and *Haplosporangium parvum* failed to present Feulgen staining material.

Differences between the Gram-acetone method and the Weigert-aniline xylene-Gram variant were generally in the direction that the latter yielded more Gram-positive organisms. *Eimeria stiedae,* almost completely Gram negative by the acetone technique, regularly contained numerous conspicuous Weigert-positive granules. One-fourth to two-thirds of the cells of *Histoplasma capsulatum* in any given preparation were Gram positive by the Weigert method, while all were negative by the acetone method. Haplosporangium, Torula, and Coccidioides all showed many more Gram-positive cells with the Weigert method. *Klossiella muris* often presents Gram-positive cells by the Weigert method, seldom with the acetone method. Encephalitozoon is weakly positive by the acetone method, while negative and strongly positive cells are seen side by side in Weigert preparations. Mycetomal and other thick mycelial fungi often present some positive filaments with the Weigert method and none with the acetone. Weigert-positive, Gram-negative material is seen in the shells of Schistosome eggs and in the opercular plugs of Capillaria eggs.

Bauer-positive polysaccharides digestible with ptyalin and malt diastase are present in the cytoplasm of *Endamoeba histolytica, Eimeria stiedae,* most Sarcosporidia, and in the egg cytoplasm of *Capillaria hepatica.*

Bauer-positive material which resists digestion by ptyalin and malt diastase forms the capsules of the following: certain yeasts, such as *Monilia candida, Histoplasma capsulatum, Torula histolytica, Haplosporangium parvum, Coccidioides immitus,* and Blastomyces species; certain coarsely mycelial fungi, such as those of mycetoma, Penicillium, Aspergillus, and others; the shells of the eggs of *Schistosoma mansoni* and *Schistosoma japonicum;* and the opercular plugs of the eggs of *Capillaria hepatica.* Bauer-positive, relatively enzyme-resistant granules are found in some Sarcosporidia, in a few individuals of *Klossiella muris,* and in Toxoplasma.

The ray fungi possess small quantities of diffuse material reacting to the Bauer test, which often appears more definitely filamentous after enzyme digestion tests. Similar bacillary and occasionally filamentous Bauer-positive material was found among the staphylococci in the granules in a case of botryomycosis. Some Gram-positive diphtheroid bacilli are also Bauer positive, with or without diastase digestion.

The Bauer method offers a valuable aid in detection of scarce amebae and yeastlike fungi in tissues. It gives structural details in the more coarsely mycelial fungi which are not apparent with other usual staining methods. It clearly demonstrates the remaining fragments of Schistosome eggs in granulomata in which they are not readily identifiable by other means.

In its possession of a Bauer-positive, enzyme-resistant capsule and in its partially Gram-positive reaction Encephalitozoon shows a certain resemblance to Coccidioides and to Histoplasma. Wenyon[10] has stated that "in many respects it resembles a small yeast, but reproduction by budding has not been observed." He further states that "It seems premature to conclude that the organism is

even a protozoon.'' The present data tend to accentuate the doubt of the protozoal nature of this organism. It is noteworthy that both Coccidioides and Histoplasma were first thought to be protozoal.

REFERENCES

1. Bauer, H.: Mikroskopisch-chemischer Nachweis von Glykogen und einigen anderen Polysacchariden, Ztschr. f. mikr.-anat. Forsch. **33**: 143-160, 1933.
2. Bensley, C. M.: Comparison of Methods for Demonstrating Glycogen Microscopically, Stain Technol. **14**: 47-52, 1939.
3. Lillie, R. D., and Greco, J.: Malt Diastase and Ptyalin in Place of Saliva in the Identification of Glycogen, Stain Technol. In press.
4. de Tomasi, J. A.: Improving the Technic of the Feulgen Stain, Stain Technol. **11**: 137-144, 1936.
5. Stowell, R. E.: Feulgen Reaction for Thymonucleic Acid, Stain Technol. **20**: 45-58, 1945.
6. Lillie, R. D.: The Gram Stain. I. A Quick Method for Staining Gram-Positive Organisms in the Tissues, Arch. Path. **5**: 828-834, 1928.
7. Perrin, T. L.: Toxoplasma and Encephalitozoon in Spontaneous and in Experimental Infections of Animals, Arch. Path. **36**: 568-578, 1943.
8. Meriwether, L. S.: Identification of Amoebae in Tissue, J. Tech. Methods **14**: 64-65, 1935.
9. Kernohan, J. W.: A New Staining Method to Demonstrate Pathogenic Yeasts and Fungi, J. Neuropath. & Exper. Neurol. **2**: 95-98, 1943.
10. Wenyon, C. M.: Protozoology, New York, 1926, William Wood & Company, pp. 754-756.

A SIMPLE METHOD OF STAINING MALARIA PARASITES

GEORGE JOANNIDES, M.D.
ATHENS, GREECE

THE report by Manwell* incites me to report another simple method for staining malarial parasites which was used during the war when no Leishman, Wright, or Giemsa's stains were available and when buffered or distilled water could not be obtained.

In a 50 c.c. porcelain evaporating dish, place 0.5 Gm. methylene blue and 1.5 Gm. borax. Add, with stirring, 30 c.c. of boiling tap water. Place the evaporating dish over a small flame at such a height as to maintain a temperature of 75 to 80° Centigrade. The liquid evaporates almost to dryness in approximately one-half hour. Add 30 c.c. of boiling tap water for a second and a third time, evaporating after each addition. Set aside for twenty-four hours. A dry blue compact mass remains in the evaporating dish. Add, without stirring, 30 c.c. of 95 per cent alcohol. After five minutes pour the alcohol into a 100 c.c. bottle and let the residue in the evaporating dish dry almost completely. Repeat the extraction in the same way, once with 30 c.c. and once with 40 c.c. of alcohol. The total alcoholic extract (about 100 c.c.) is blue and contains no precipitate.

On the unfixed blood film, place 15 drops of the alcoholic stain. After thirty to sixty seconds add 15 drops of a 1 to 2000 solution of eosin in tap water. Mix the stains on the slide by means of a glass rod. After three to four minutes flood the slide with tap water, wash with tap water, and let dry. The red cells appear light green with a pinkish cast, the eosinophile granules stain pink, and the remaining leucocytes and the platelets stain as with Giemsa's stain. The malarial parasites also have the same appearance as when treated with Giemsa's stain but are stained somewhat less deeply.

The satisfactory results obtained by this method are probably due to: (1) nearly complete transformation of methylene blue to azure; (2) the greater solubility in alcohol of the azure as compared to the methylene blue solubility; and (3) the insolubility of borax in alcohol. The technique constitutes a combination and modification of older ones published by many authors.

From the Pasteur Institute.
Received for publication, Sept. 23, 1946.

*Manwell, R.: The J. S. B. Stain for Blood Parasites, J. LAB. & CLIN. MED. 30: 1078, 1945.

THE USE OF RUSSELL VIPER VENOM AS A MEANS OF FOLLOWING THE ANTICOAGULANT ACTION OF 3,3′-METHYLENEBIS (4-HYDROXYCOUMARIN) IN THE DOG

EDWIN J. DE BEER
TUCKAHOE, N. Y.

WITH THE TECHNICAL ASSISTANCE OF ROCCO FANELLI

FULLERTON,[1] Page and Russell,[2] Shapiro and co-workers,[3] and others have shown that when Russell viper venom was substituted for thromboplastin in Quick's[4] prothrombin time test, certain points of resemblance could be noted. The purpose of this paper is to present comparative data obtained by the action of each of the two clotting accelerators on the plasma of dogs treated with 3,3′-methylene bis (4-hydroxycoumarin) (Dicumarol).*

Link and associates[5, 6] have shown that dicumarol is probably responsible for the hemorrhagic action of spoiled sweet clover hay. It is believed generally that the drug acts by reducing the effective prothrombin level. In fact, Quick's prothrombin time test was used to guide the isolation work. Without attempting to review the literature in the clinical field, it may be stated also that thromboplastin has been frequently employed to follow the course of dicumarol therapy.[7-9] Russell viper venom also has been used for this purpose, and among its users may be mentioned the names of Shapiro and Sherwin[8] and Wright and Prandoni.[10]

METHODS

Blood was obtained from dogs by venipuncture, decalcified by mixing with 2 mg. of dry potassium oxalate per cubic centimeter of blood, and centrifuged promptly. The clear plasma was diluted by adding 3 volumes of 0.9 per cent NaCl and the prothrombin time determined on one portion with 2.5 per cent thromboplastin as described by Quick.[4] A commercial preparation prepared from rabbit brain was used for this purpose. This concentration of plasma (25 per cent) was found to be well suited for use with Russell viper venon since it yielded times of convenient length. Much longer times were obtained with thromboplastin at the maximum dicumarol effect. It may be noted that times may be shortened for both thromboplastin and Russell viper venom by increasing the plasma concentration or they may be lengthened by diluting the plasma.

A second portion was tested with Russell viper venom instead of thromboplastin. In carrying out this determination, 0.1 c.c. of the 25 per cent plasma was mixed with 0.1 c.c. of 1:10,000 Russell viper venom† and 0.1 c.c. of 1 per

From the Wellcome Research Laboratories.
Received for publication, June 17, 1946.

*Dicumarol is the trademark for a commercial preparation of 3,3′-methylene bis (4-hydroxycoumarin).

†Supplied as Stypven Brand Russell Viper Venom by Burroughs Wellcome & Co. (U. S. A.) Inc., New York, N. Y.

cent calcium chloride was then added. A stop watch was started at the instant of the addition of the calcium chloride and stopped when the first flecks of fibrin appeared. For the sake of convenience this elapsed time interval will be known as the RV time. All determinations were made in triplicate. Eight different dogs were used in these experiments.

Dicumarol in gelatin capsules was given orally.

RESULTS

The results of a typical experiment are given in Table I. It will be observed that the RV times paralleled the prothrombin times in several respects. Both coagulating agents simultaneously revealed the onset of dicumarol action, both indicated about the same duration of action, and both showed peaks of maximum intensity at about the same time. It will be observed that the thromboplastin figures rose to relatively higher values. Also thromboplastin figures fell to relatively lower levels during the reaction period which lasted for two or three weeks after the dicumarol administration.

TABLE I. RELATIONSHIPS BETWEEN DICUMAROL ADMINISTRATION, PROTHROMBIN TIME, AND RV TIME IN DOG

DATE	MEAN RV TIME (SECONDS)	MEAN PROTHROMBIN TIME (SECONDS)	DICUMAROL DOSAGE (MG. PER KG.)
11/15	28.3	31.1	10
11/16	40.5*	57.3*	10
11/17	46.6*	314.0*	10
11/18	71.4*	>900.0*	--
11/20	107.0*	>900.0*	--
11/22	62.4*	182.0*	--
11/24	40.3*	31.3	--
11/25	28.0	34.1	--
11/27	31.3	30.4	--
12/11	21.8	18.3	--
12/12	23.2	17.1	--
12/14	21.2	13.1	--
12/16	24.0	14.7	--
12/18	24.8	13.9	30
12/19	38.8*	26.9*	--
12/20	49.8*	56.3*	--
12/21	50.3*	121.0*	--
12/22	56.3*	433.0*	--
12/23	61.8*	133.0*	--
12/26	34.5*	20.2	--
12/27	29.2	17.8	--
12/29	26.6	17.7	--
1/1	29.8	20.1	--

All determinations were done in triplicate on 25 per cent dog plasma.
*Values significantly above the normal level.

These experiments confirmed the earlier observations of Butt and associates[7] that divided doses of dicumarol were more effective than the same quantity administered in a single dose.

In considering the significance of the effects produced by dicumarol, account must be taken of the reproducibility of the test and the fluctuations in normal levels. Both thromboplastin and Russell viper venom gave highly consistent values. The total range experienced in triplicate determinations is rarely more than one or two seconds. Normal times, however, vary from

dog to dog, and Fig. 1 gives an example of how times may vary within the same animal. If the data of November 15, 25, 27, December 11 to 18, 27, 29, and January 1, as given in Table I, be considered as normal values, it will be seen that the RV times for this animal varied about a mean of 26.2 seconds. The standard deviation of these eleven values was 3.4 seconds. It is commonly assumed that any sample which breaks through a range embraced by the mean ± 2 times the standard deviation should be considered significantly different. In this case the upper limit would be 26.2 + (2 × 3.25) or 32.7 seconds. If this figure be taken as a guide, it is apparent that the administrations of dicumarol were followed by significant increases in RV times.

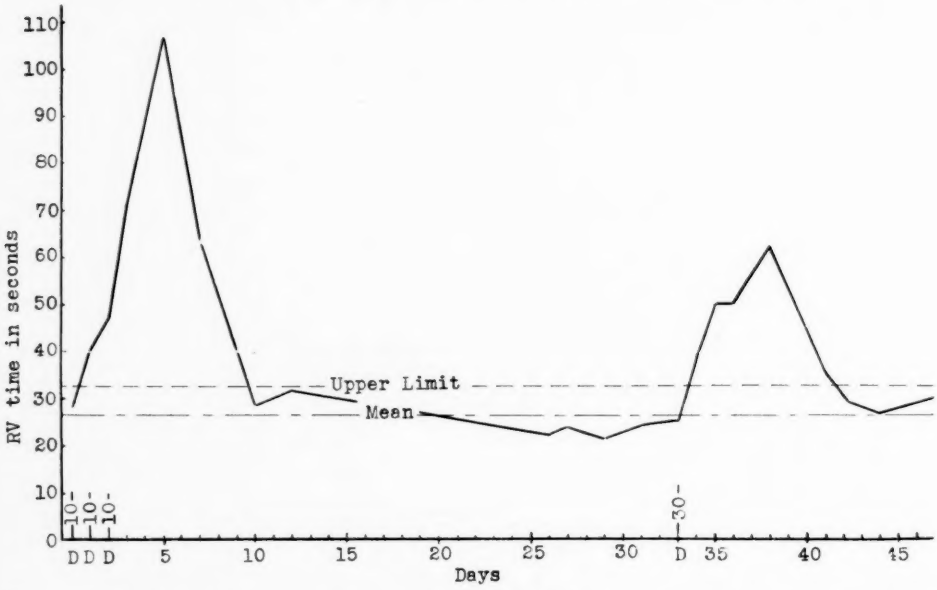


Fig. 1.—The effect of dicumarol on the RV time of dog plasma. *D*, Dicumarol in milligrams per kilogram.

SUMMARY

Russell viper venom resembled thromboplastin in its ability to follow the course of the anticoagulant action of 3,3′-methylene bis (4-hydroxycoumarin) in the dog.

The times for the formation of clots by thromboplastin were relatively longer than the times for the formation of fibrin flecks by Russell viper venom.

REFERENCES

1. Fullerton, H. W.: Estimation of Prothrombin; a Simplified Method, Lancet 2: 195, 1940.
2. Page, R. C., and Russell, H. K.: Prothrombin Estimation Using Russell Viper Venom. I. Simple Modification of Quick's Method, J. Lab. & Clin. Med. 26: 1366, 1941.
3. Shapiro, S., Sherwin, B., Redish, M., and Campbell, H. A.: Prothrombin Estimation; a Procedure and Clinical Interpretations, Proc. Soc. Exper. Biol. & Med. 50: 85, 1942.
4. Quick, A. J.: The Hemorrhagic Diseases and the Physiology of Hemostasis, Springfield, Ill., 1942, Charles C Thomas, Publisher.

5. Campbell, H. A., and Link, K. P.: Studies on the Hemorrhagic Sweet Clover Disease. IV. The Isolation and Crystallization of the Hemorrhagic Agent, J. Biol. Chem. **138**: 21, 1941.
6. Stahmann, M. A., Heubner, C. F., and Link, K. P.: Studies on the Hemorrhagic Sweet Clover Disease. V. Identification and Synthesis of the Hemorrhagic Agent, J. Biol. Chem. **138**: 513, 1941.
7. Butt, H. R., Allen, E. V., and Bollman, J. L.: A Preparation From Spoiled Sweet Clover [3,3′-Methylene-bis-(4-hydroxycoumarin)] Which Prolongs Coagulation and Prothrombin Time of the Blood; Preliminary Report of Experimental and Clinical Studies, Proc. Staff Meet., Mayo Clin. **16**: 388, 1941.
8. Shapiro, S., and Sherwin, B.: Studies in Thrombo-Embolization. II. Observations on the Use of Dicumarol (3,3′ Methylenebis [4-hydroxycoumarin]) in Embolization. Report of 5 Cases, New York State J. Med. **43**: 45, 1943.
9. Hurn, M., Barker, N. W., and Magath, T. B.: Prothrombin Time Determinations Following the Administration of Dicumarol With Special Reference to the Variability of Thromboplastin, Proc. Staff Meet., Mayo Clin. **19**: 507, 1944.
10. Wright, I., and Prandoni, A.: The Dicoumarin 3,3′-Methylene-bis-(4-hydroxycoumarin); Its Pharmacologic and Therapeutic Action in Man, J. A. M. A. **120**: 1015, 1942.

MICROANALYSIS OF OPIATES BY X-RAY DIFFRACTION*

S. T. Gross,† Urbana, Ill., and Fred W. Oberst,‡ Lexington, Ky.

SINCE the development of various derivatives of morphine and the ultimate study of many of them in man and animals, it has become more difficult to find chemical tests suitable for their identification and determination in amounts present in biologic materials after administration. Occasionally toxicologic examinations of the lesser known opium derivatives require other than chemical means for a specific and conclusive identification.

The x-ray diffraction method has been used successfully for many years as a critical, characteristic means of identification, but it has not found application in many fields where only extremely small amounts of materials are available for analysis. Its advantages have led us to develop microdiffraction techniques of sufficient sensitivity that practically any sample of crystalline material may be identified and determined quantitatively. The new methods of identification serve not only to adapt small samples for diffraction information but include topochemical reactions in the preparation and examination of derivatives.

METHODS

The flat cassette method of x-ray diffraction was employed in the present experiments (Fig. 1). The x-ray beam, collimated to a thin pencil of radiation about 0.010 inch in diameter, was allowed to penetrate the sample, and the diffraction pattern was recorded on a flat film mounted perpendicularly to the x-ray beam and separated from the sample by 5 centimeters. The sample holder was a small metal disk perforated with an opening $\frac{1}{64}$ inch in diameter in which the sample (about 0.08 mg. or 80 γ) was packed. X-radiation from a copper target diffraction tube, filtered through 0.001 inch nickel foil, was used; exposures were two hours at 14 ma. and 40 kv. peak. Data are tabulated in terms of interplanar spacings or "d" values, where "d" is defined by the Bragg law,

$$d = \frac{\lambda}{2 \sin\Theta}.$$

The wave length of x-radiation used (λ) is essentially 1.54 A.U.; 2 Θ represents the angular deviation of the diffracted x-ray beam from the undiffracted beam (Fig. 1). Intensity values were estimated with a Leeds and Northrup recording microphotometer. No attempt was made to correct for absorption of the x-ray

Received for publication, Sept. 19, 1946.

*The x-ray work was carried out in the Department of Analytical Chemistry at the University of Illinois. The drugs used in the test were prepared by Dr. Lyndon F. Small, formerly of the University of Virginia and now at the National Institute of Health, while participating in the work of the Committee on Drug Addiction of the National Research Council.

†Formerly with the Noyes Laboratory of Chemistry, University of Illinois; now with the General Aniline and Film Corporation, Easton, Pa.

‡Formerly Biological Chemist, Research Department, United States Public Health Service Hospital; now with the Wm. S. Merrell Company, Cincinnati, Ohio.

beam in the samples, although samples were made as thin as possible to obviate this factor. Intensity values listed are comparable only with patterns made by the flat cassette method, although "d" values are identical within experimental error with those obtained from any type of x-ray powder diffraction pattern technique.

A single pattern is acceptable identification when exact coincidence with a reference is obtained, but the use of two patterns of an alkaloid (one of the base and one of its hydrochloride salt) renders the result conclusive. Such reactions are readily carried out topochemically as follows: After a pattern of the free base is obtained, the mounted sample may be exposed to hydrochloric acid fumes for a short time. This converts the base to the hydrochloride salt (as shown by the absence of the diffraction lines of the base), and the second diffration pattern may be obtained. If the starting material were the hydrochloride, exposure to ammonia fumes will convert it to the free base.

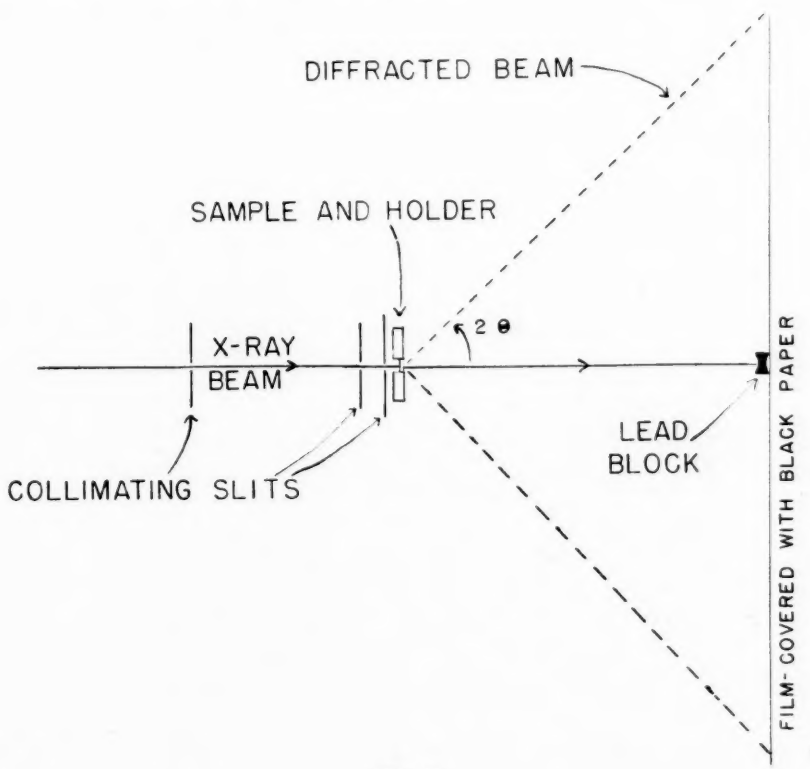


Fig. 1.

These gas treatments are conveniently carried out by rinsing a beaker with concentrated hydrochloric acid or with ammonium hydroxide, as desired, and inverting it over the sample mounted in the holder for twenty to thirty minutes. The sample holder must be constructed of material which will not react with the fumes; otherwise, it is necessary to remove the sample from the holder and

replace it later. The pattern of ammonium chloride formed in the sample will appear on subsequent diffraction patterns, but does not interfere with the identification.

RESULTS

X-ray Diffraction Patterns of Several Opiates and of Demerol.—The data given in Table I are arranged for classification according to the system employed by Hanawalt[1] in his tables for the identification of unknown materials by the diffraction method. Roman numerals I, II, and III, following intensity values, correspond to the three strongest lines in the order of their intensity. Symbol B indicates that the line is unusually broad, and brackets are employed when two lines are so close together that slight changes in experimental conditions might make them appear as a single diffraction interference.

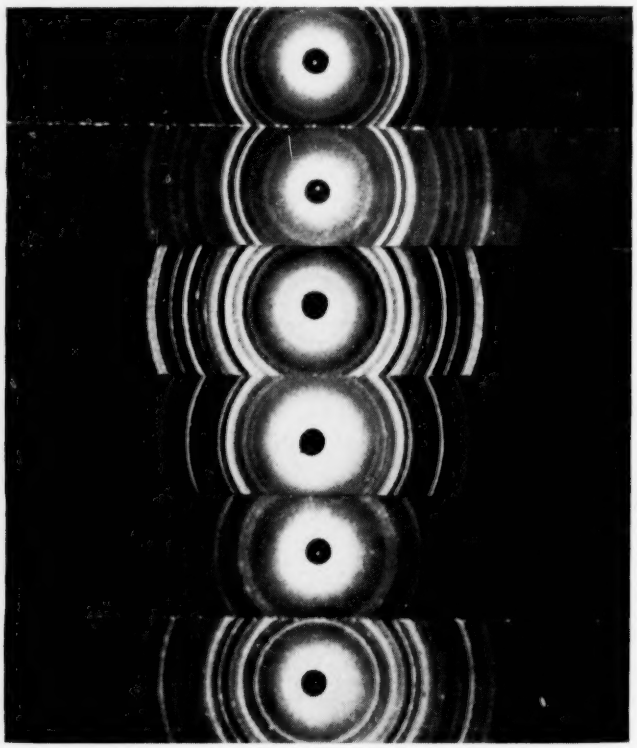


Fig. 2.—Patterns made with filtered copper K-alpha radiation; sample—film distance, 5 cm.; two-hour. exposure, 20 Ma. and 40 kv. *A*, morphine base; *B*, morphine hydrochloride; *C*, morphine sulfate dihydrate; *D*, morphine sulfate pentahydrate; *E*, heroin base; *F*, heroin hydrochloride.

The characteristic experimental measurements for a number of opiates and for Demerol* are listed in Table I; example prints are shown in Fig. 2. Attention is directed to the patterns of two hydrates of morphine sulfate, the one be-

*This drug was furnished by Dr. O. W. Barlow, formerly of the Winthrop Chemical Company, Inc., New York, N. Y.

ing a dihydrate and the other a pentahydrate.* The pattern obtained after heating the pentahydrate of morphine sulfate between 185 and 195° C. for two hours is also included in the table. This pattern is reproducible and probably represents largely the anhydrous form, although it may also contain some other decomposition products. This pattern is not accounted for by combination of the two hydrates.

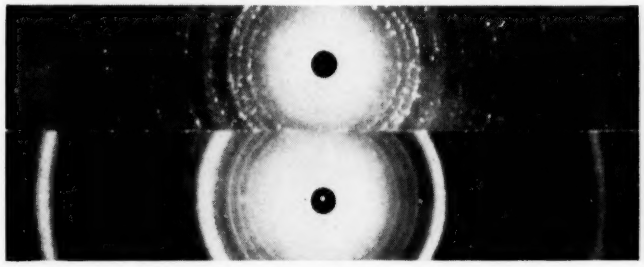


Fig. 3.—*A*, Diffraction pattern of urinary residue after heroin administration, showing presence of morphine (small amount of magnesium oxide added). Exposure made seven hours. *B*, Diffraction pattern of 0.7 γ morphine suspended in 0.06 mg. magnesium oxide.

Detection of Morphine in Urine by X-ray Method.—In Fig. 3*A* is shown the diffraction pattern of an alkaloid present in a residue prepared from urine of an addict receiving heroin.† Comparison with Fig. 3*B* shows that this product is largely morphine; no heroin could be detected. This is in accord with excretion studies previously reported.[3] The total amount of sample available in this case was about 0.98 milligram. The spots on the pattern indicate that the material was present as crystals with particle size greater than about 10^{-3} centimeters. Below this dimension the lines are sharp and smooth until colloidal dimensions are attained. In either case the position of the diffraction lines is unchanged.

Since urinary residues containing morphine, etc., are rather hygroscopic, they should be examined under conditions of reduced humidity.

DISCUSSION

The described method of identification by x-ray diffraction technique is accepted as definite, especially when based upon two diffraction patterns. It is not

*The results on two hydrates of morphine sulfate are worthy of mention. Patterns obtained from a single commercial sample were not identical with those of Dr. Howard L. Andrews of the National Institute of Health. This stimulated further examination of seven additional samples; three of these gave the same pattern as ours and four were identical with those of Dr. Andrews.

Dr. Andrews[5] heated the pentahydrate of morphine sulfate at 57° C. for two hours and then made an x-ray pattern in a sealed tube. A mixture of the two patterns was obtained. Heating the pentahydrate at 60° C. for two hours gave a compound having a pure pattern agreeing with our original one. When the tube was left open for a few hours, the diffraction pattern returned to the form found for the pentahydrate. Weight loss determinations made by Dr. Andrews indicate that the change in patterns is accompanied by a loss of 3 molecules of water. It is believed that one pattern is obtained from the pentahydrate and the other from the dihydrate.

†The alkaloid excreted in urine was extracted by means of a chloroform-alcohol mixture (3 and 1 parts, respectively) according to the technique described by Oberst,[2] using a liquid-liquid extractor. After evaporation of the solvent the residue was subjected to isoelectric extractions. The residue obtained after evaporation of the organic solvent was then treated with permutit to separate morphine from undesirable substances extracted. The permutit was washed and then treated with alkali to remove morphine which was re-extracted with the chloroform-alcohol mixture. After evaporation of the solvent the residue was weighed and submitted to x-ray examination.

TABLE I. INTERPLANAR SPACINGS AND RELATIVE INTENSITIES,* USING A FLAT CASSETTE AND FILTERED COPPER K-ALPHA RADIATION

d A.U.	I	d A.U.	I
1. Morphine Base			
10.14	0.16	4.82	0.15
7.37	0.50 III	4.16	0.45
6.59	0.65 II	3.98	0.10
6.25	0.15	3.71	0.10
6.00	1.00 I	3.42	0.30
5.00	0.50		
2. Morphine Hydrochloride			
10.65	0.15	4.61	0.48
7.91	0.15	4.11	0.60
6.70	1.00 I	3.86	0.45
5.99	1.00 II	3.57	0.30
5.68	0.60 III	3.38	0.50
4.79	0.30		
3. Morphine Sulfate Dihydrate			
14.69	0.10	3.84	0.20
7.99	0.08	3.53	0.25
7.20	0.50 II	3.41	0.25
6.64	0.13	3.19	0.05
6.24	1.00 I	3.10	0.08
5.47	0.20	2.96	0.02
5.19	0.10	2.86	0.04
4.97	0.15	2.71	0.02
4.69	0.10	2.64	0.04
4.27	0.30 III	2.50	0.00
4. Morphine Sulfate Pentahydrate			
15.10	0.35	4.36	0.80 II
8.35	0.10	4.06	0.05
7.65	0.15	3.86	0.05
7.16	0.15	3.69	0.05
6.21	1.00 I	3.39	0.45
5.64	0.50 III	3.26	0.05
5.24	0.10	3.12	0.15
5.01	0.20	2.63	0.15
4.70	0.05		
5. Morphine Sulfate, Probably Anhydrous (The pentahydrate was heated 185 to 195° C. for two hours)			
15.15	0.5	4.26	0.5
13.46	0.7 III	3.98	0.2
7.91	0.3	3.61	0.2
7.25	0.1	3.41	0.1
6.30	1.0 I	3.35	0.1
5.86	0.6	3.22	0.1
5.51	0.2	3.14	0.1
5.09	0.8 II	2.94	0.2
4.71	0.1	2.70	0.2
4.55	0.1	2.47	0.1
		2.32	0.1
6. Codeine Base			
6.53	1.00 I	3.49	0.10
6.04	0.10	3.23	0.10
5.23	0.33	3.02	0.10
4.89	0.46 II	2.85	0.10
3.89	0.22	2.70	0.10
3.75	0.45 III		
7. Codeine Hydrochloride			
10.33	0.50	3.91	1.00 I
8.04	0.80 III	3.75	0.20
6.11	0.71	3.29	0.20
5.70	0.90 II	3.09	0.20
5.15	0.30	2.97	0.20
4.71	0.25 B	2.77	0.20

TABLE I—CONT'D

d A.U.	I	d A.U.	I
8. Dilaudid (Dihydromorphinone. May contain some NH_4Cl.)			
10.41	0.29	4.59)	0.58 B
9.62	0.40	4.43)	
7.15	1.00 I	4.15	0.20
6.62	0.75 II	3.78	0.15
6.03	0.60 III	3.58	0.48
5.54	0.30	3.41	0.20
5.15	0.15	2.64	0.10
4.83	0.10		
9. Dilaudid Hydrochloride			
9.50	0.10	4.36	0.20
6.50	1.00 I	4.07	0.50
6.04	1.00 II	3.50	0.50
5.56	0.50 III	3.37	0.50
5.01	0.20	3.02	0.15
4.72	0.15	2.69	0.25
4.51	0.50		
10. Heroin Base (Diacetylmorphine)			
7.95)	0.75 III	4.56	0.25
7.48)		4.30	0.60
6.99	0.80 II	3.85	0.40
5.51	1.00 I	3.53	0.50
4.96	0.30 B	3.22	0.20
11. Heroin Hydrochloride			
18.35	0.17	4.63	0.20
9.19	0.70	4.47	0.20
8.04	0.40	4.26	0.30 B
7.18	1.00 I	4.01	0.70
6.84	0.15	3.57	0.30
6.38	0.75	3.36	0.50
5.79	0.90 II	3.18	0.25
5.33	0.20	3.01	0.10
5.13	0.90 III	2.63	0.10
4.91	0.20	2.37	0.10
12. Monoacetylmorphine Hydrochloride			
10.97	0.15	4.34	0.26
8.37	1.00 I	4.10)	0.30
6.61	0.50	3.99)	
6.11	0.60 II	3.59	0.50
5.92	0.60 III	3.34	0.15
6.62	0.10	3.21	0.25
5.30	0.15	3.05	0.20
5.06	0.40	2.93	0.10
4.70	0.35	2.81	0.10
13. Metopon (Methyldihydromorphinone hydrochloride)			
6.90	1.00 I	3.80	0.20
6.45	0.90 II	3.46	0.30
6.02	0.70	3.15	0.10
5.56	0.30	2.92	0.10
5.10	0.30	2.77	0.10
4.67	0.50	2.71	0.10
4.43	0.20		
4.17	0.85 III		
14. Demerol (Hydrochloride of 1-methyl-4-phenyl-piperidine-4-carboxylic acid ethyl ester)			
7.79	1.00 I	3.70	0.12
6.78	0.22	3.53	0.80 III
5.61	0.70	3.35	0.10
5.16)	0.80 II	3.26	0.10
5.03)		3.00	0.10
4.59	0.62	2.81	0.10
3.99	0.50	2.54	0.10

After the numbers, I, II, and III represent the three strongest lines in order of their intensity; B indicates an unusually broad line; and brackets are used to indicate two lines which are barely resolved.

limited to alkaloids; any crystalline material may be so identified. Patterns of a number of alkaloids have been prepared by one of us (S. T. G.), and the topochemical method of preparing salts, or the free bases, works quite as well as with the opium alkaloids shown in Table I. Such identifications are of definite toxicologic interest, since the material used is not consumed, the identity is certain, and the diffraction patterns serve as permanent records.

It is possible to obtain diffraction patterns with extremely small amounts of material by using a small sample holder and a very thin powdered sample. Even smaller samples may be used if some inert material, comparatively transparent to the radiation, is added to support the alkaloid. With such a mixture the morphine pattern has been obtained with as little as 0.0007 mg. (0.7 γ) of morphine in the sample. Only the strongest lines are present in patterns containing such small amounts of alkaloid (Fig. 3*B*). It is necessary that the mounted sample contain at least 1 per cent of morphine; hence, overdilution with the inert material must be avoided. Such a sample was prepared by mixing the free alkaloid in an aqueous suspension with a weighed amount of magnesium oxide to give a thorough dispersion of the insoluble alkaloid. After drying, a small amount of the weighed mixture was used for the sample mount. Reaction between the magnesium oxide and water is quite negligible.

Experiments were carried out by precipitating the base with ammonium hydroxide fumes from a solution of an alkaloidal salt in the presence of a suspension of magnesium oxide. This method is also satisfactory, but diffraction lines from addition products, which must be recognized and discounted, complicate the pattern.

By addition of some known material in a definite proportion to a sample, it is possible to test for the alkaloid quantitatively. Such a quantitative microestimation requires a comparison of line intensities and a correction factor. As a description of this method has been published,[4] details will not be given here. The flat cassette type of pattern is suitable for such an analysis, which makes it possible to determine all important constituents of a mixture from one diffraction pattern, provided the necessary reference data are available.

Advantages of the x-ray diffraction technique over other methods of identification are: (1) characteristic and specific diffraction patterns are obtained for any given substance, (2) employment of extremely small amounts of material is possible, (3) a permanent record is obtained, (4) the sample is not destroyed, (5) samples need not be pure (only a small percentage of the material being necessary for identification in the absence of heavy absorbing inorganic salts), (6) all of the materials may be identified in mixtures, although the analysis is more tedious than in the case of a simple compound, and (7) quantitative estimation is possible with a very small change in procedure. Among the disadvantages of this technique, mention should be made of the fact that the presence of either solid solutions or colloids may give unsatisfactory results; however, this condition was not encountered with the materials used in the present study. It is necessary to have standard patterns, or data such as are given in this paper, to carry out the identification.

SUMMARY

A micromethod for a conclusive identification of various alkaloids, either singly or in mixtures, is described. The specific data necessary for identification of a number of the opium alkaloids are listed in a suitable form for general usage. The sensitivity of the method has been shown to be sufficient to detect and identify quantities of alkaloid as small as 0.0007 mg. (0.7 γ).

A method is described for converting the free base of an alkaloid to a salt in the sample holder without loss of material, whereby two diffraction patterns, rather than one, are used to make identification conclusive.

The authors wish to acknowledge their indebtedness to Dr. Lyndon F. Small, for supplying many of the opiates studied, and to Dr. Howard L. Andrews and Dr. Small, for reading the manuscript and offering helpful suggestions.

REFERENCES

1. Hanawalt, J. D., Rinn, H. W., and Frevel, L. K.: Chemical Analysis by X-ray Diffraction, Indust. & Engin. Chem. (Anal. Ed.) **10**: 457, 1938.
2. Oberst, F. W.: The Determination of Morphine in the Urine of Morphine Addicts, J. LAB. & CLIN. MED. **24**: 318, 1938.
3. Oberst, F. W.: Studies on the Fate of Heroin, J. Pharmacol. & Exper. Therap. **79**: 266, 1943.
4. Gross, S. T., and Martin, D. E.: Quantitative Determination of Crystalline Materials by X-ray Diffraction, Indust. & Engin. Chem. (Anal. Ed.) **16**: 95, 1944.
5. Andrews, H. L.: Personal communication.

A METHOD OF BONE MARROW BIOPSY FROM THE RAT*

I. Myron Vigran, M.D.†
Rochester, Minn.

IN STUDIES of experimental anemia it is frequently essential to observe cytologic changes which occur within the bone marrow. In connection with a recent study of the effects of synthetic folic acid (folvite) on certain experimentally induced anemias, some procedure which permitted frequent sampling of the bone marrow in individual rats was essential. Since this procedure has proved most satisfactory and since I am aware of but one published method‡ for obtaining specimens of bone marrow from small animals, I believe that a report of this technique may be of interest to hematologists.

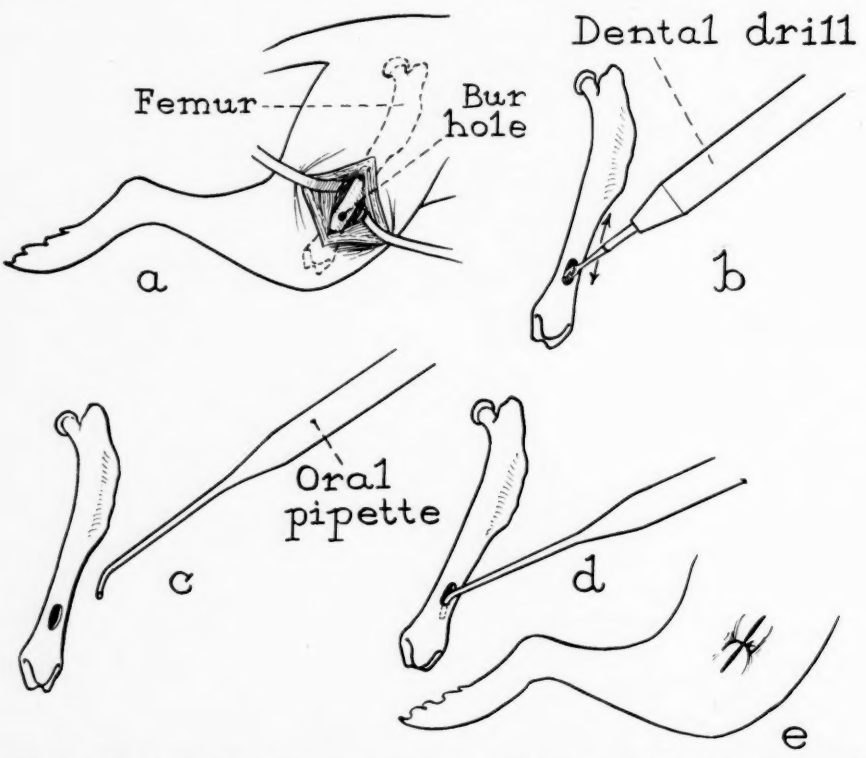


Fig. 1.—Technique of obtaining bone marrow. *a*, Incision of skin and fascia, separation of fibers of biceps, and opening in femur. *b*, Enlargement of opening. *c*, Pipette. *d*, Insertion of pipette. *e*, Closure of incision.

The procedure may be performed quickly, usually requiring less than ten minutes. Anesthesia is induced with an intraperitoneal injection of pentobar-

Received for publication, Nov. 2, 1946.

*Work done in the Division of Experimental Medicine, Mayo Foundation, under the direction of Dr. George M. Higgins.

†Fellow in Medicine, Mayo Foundation.

‡Steinberg, B., and Martin, R. A.: Removal of Bone Marrow in Living Animals, Proc. Soc. Exper. Biol. & Med. 61: 428, 1946.

bital sodium in amounts equal to 30 mg. per kilogram of body weight. Ether anesthesia may also be used. The rat is placed on an operating board in a prone position and the hair over the lateral aspect of the thigh is clipped. An incision approximately ½ inch (1.3 cm.) in length is made through the skin and fascia directly over the distal third of the femur. The fibers of the short head of the biceps muscle are then carefully separated by means of a small hemostat, thereby exposing the femur. The muscle fibers on either side of the bone are retracted with small aneurysm needles and the periosteum of the femur is stripped away. Using a crosscut dental bur (No. 560), a small opening is made through the cortex into the marrow cavity (Fig. 1*a*). The circular opening is then slightly enlarged in the direction of the longitudinal axis of the bone, forming an oblong aperture which readily permits the insertion of the tip of an especially designed pipette into the marrow space (Fig. 1*b*). The pipette, which previously has been rinsed with a solution of heparin, is passed through the opening so that its tip lies in the marrow space parallel to the long axis of the bone (Fig. 1*c* and *d*). A small quantity of the marrow is then orally aspirated. Appropriately sized drops are placed on clean glass slides and films are made in the usual manner.

The incision is closed with a single cotton suture extending through the skin into the deep muscles (Fig. 1*e*). Untoward aftereffects are seldom noted, and the incision is usually well healed within a week. The slides are stained with MacNeal's tetrachrome stain. The procedure may be repeated within a week on the opposite femur and within three weeks on the original one.

THE USE OF DRIED ANTIGEN PREPARATIONS FOR TESTING PNEUMOCOCCUS TYPING SERA

JANET M. BOURN, PH.D., AND CHARMION M. CARNES, A.B.

THE testing of pneumococcus typing sera for specific and cross reactions according to the requirements of the National Institute of Health served to stimulate the study reported here. The problem was to find a way of preserving pneumococcus typing antigens for a period longer than four days and to demonstrate the successful use of such antigens.

The directions for the preparation of pneumococcus antigens, as given by the National Institute of Health,[1] may be summarized as follows: Each antigen used is a young (one to five hour), fully virulent broth culture to which 1.43 per cent commercial formalin has been added and which has been checked for capsular swelling and agglutination with homologous antiserum. The antigens should be kept in a cold room when not in use and in an iced container when in use. The antigen may be used as long as the pneumococci stay in suspension and the swollen capsules appear intact and are not fragmented, usually a period of three or four days.

The use of dried antigen preparations as a means of preserving pneumococcus antigens for a period longer than four days was selected as feasible and was tested in the following manner: The thirty different types of pneumococcus which were used for such antigens represented strains which had been received from the National Institute of Health, except types 5 and 7 which were obtained from the New York City Department of Health. The antigens were prepared from Felton broth cultures which had been incubated four or five hours at 37° Centigrade. A small loopful (28 gauge platinum loop, 1 mm. inside diameter) of each living antigen was placed on a new clean microscopic slide as in making a Neufeld preparation, and the same sized loopful of purified gastric mucin was mixed well with the antigen but confined to a small area. This was allowed to dry in the air, after which the preparations were stored in regular wooden slide boxes free from dust. Mucin was added as a protective medium for the pneumococci with the idea that the specificity of the dried organisms might be demonstrated over a longer period than otherwise. However, it was found necessary to purify the gastric mucin following the method of Chase,[2] since the crude product contained an amorphic material which interfered with the Neufeld test.

Similar dried preparations were made, using pneumococcus antigens types 1, 2, and 3, which had been formalinized according to the National Institute of Health requirements. In addition, four serial sets of dried living preparations not containing formalin were made for testing cross reactions of pneumococcus typing serum.

From the Bureau of Laboratories, Michigan Department of Health.
Received for publication, Nov. 9, 1946.

As shown in Table I, the thirty different types of dried pneumococcus antigens prepared with purified gastric mucin gave very good Neufeld reactions for a period of four weeks. Positive agglutination of all the different types of pneumococcus was demonstrated throughout the entire observation period of nine weeks, whereas the capsules gradually disintegrated after four weeks. The pneumococcus antigens which were formalinized, that is, types 1, 2, and 3, gave results which were comparable to those of the dried living antigens.

TABLE I. DURATION OF SPECIFICITY OF DRIED PREPARATIONS OF PNEUMOCOCCUS AS DETERMINED BY NEUFELD REACTION

TYPES	4/20/39	4/26/39	4/28/39	5/4/39	5/10/39	5/19/39	5/25/39	6/1/39	6/8/39	6/16/39
					*Dried, Living**					
1	++	++	++	++	++	++	++	++	++	++
2	++	++	++	++	++	+±	+±	+±	+−	+−
3	++	++	++	++	++	+±	+±	+±	+±	+±
4	++	++	++	++	++	+±	+±	+−	+−	+−
5	++	++	++	++	++	++	++	+−	+−	+−
6	++	++	++	++	++	++	++	++	+±	+−
7	++	++	++	++	++	++	+−	+−	+−	+−
8	++	++	++	++	++	+±	+±	+±	+−	+−
9	++	++	++	++	++	+±	+±	+−	+−	+−
10	++	++	++	++	++	+±	+±	+−	+−	+−
11	++	++	++	++	++	+±	+±	+−	+−	+−
12	++	++	++	++	++	++	++	++	+−	+−
13	++	++	++	++	++	+±	+±	+−	+−	+−
14	++	++	++	++	++	++	++	++	+±	++
15	++	++	++	++	++	++	++	+±	+−	+−
16	++	++	++	++	++	++	++	+−	+−	+−
17	++	++	++	++	++	++	+±	+±	+−	+−
18	++	++	++	++	++	++	++	+−	+−	+−
19	++	++	++	++	++	+±	++	+±	+±	+−
20	++	++	++	++	++	++	+±	+±	+−	+−
21	++	++	++	++	++	+−	+−	+−	+−	+−
22	++	++	++	++	++	+±	+±	+−	+−	+−
23	++	++	++	++	++	++	++	+−	+−	+−
24	++	++	++	++	++	+±	++	+±	+−	+−
25	++	++	++	++	++	++	++	+±	+−	+−
27	++	++	++	++	++	+±	+±	+±	+−	+−
28	++	++	++	++	++	+−	+±	+±	+−	+−
29	++	++	++	++	++	+±	+±	+−	+−	+−
31	++	++	++	++	++	+±	+±	+±	+−	+−
32	++	++	++	++	++	++	++	+±	+−	+−
					Dried, Formalinized†					
1	++	++	++	++	++	++	++	+−	+−	+−
2	++	++	++	++	++	+±	+±	+±	+−	+−
3	++	++	++	++	++	+±	+±	+±	+±	+±

++, Positive agglutination and capsular swelling.
+±, Positive agglutination and less than 90 per cent capsulation, with debris present.
+−, Positive agglutination and no capsular swelling.
*Types 1 to 15, inclusive, prepared 4/18/39; remaining types prepared 4/19/39.
†Formalinized according to National Institute of Health requirements, prepared 4/19/39.

In order to check the efficiency and dependability of the dried antigen preparations for testing pneumococcus typing sera, four such sera were tested: first, by the method required by the National Institute of Health, and second, by the same method except for substitution of dried living antigen preparations in place of formalinized antigens in suspension. After the Neufeld tests were prepared they were incubated for one-half hour at 37° C. as required by the National Institute of Health. Upon examination the dried antigen preparations were found to be as clean-cut in appearance as were the other preparations.

In summary, the dried antigen preparations made from fresh living antigens of thirty different types of pneumococcus gave good specificity tests as determined by Neufeld reactions both for capsular swelling and agglutination for a period of four weeks. After four weeks there was a gradual disintegration of the capsules resulting in a deposit of amorphic debris in the preparations. Similarly prepared formalinized antigen preparations of types 1, 2, and 3 pneumococcus gave corresponding results over the same period.

The dried preparations proved to be equally satisfactory for testing antipneumococcus typing serum. The results were equivalent to the results obtained by using the same sera and following the directions given by the National Institute of Health.

Although we were not familiar with Pranion's[3] improved technique for pneumococcus typing by the "Quellung" reaction when this study was in progress, it is interesting to comment in passing that the results obtained by Pranion are in agreement with the findings reported here.

It is also of interest in connection with this study to call attention to Eddy's[4] report of a simple procedure for detecting cross reactions in diagnostic antipneumococcic serum.

REFERENCES

1. Regulations of the National Institute of Health: Minimum Requirements for Pneumococcic Typing Serum, B-2772, Jan. 16, 1939.
2. Chase, M. W.: A Microorganism Decomposing Group-Specific A Substances, J. Bact. **36**: 383, 1938.
3. Pranion, A. B.: Improved Technique for Pneumococcus Typing by the "Quellung" Reaction, J. LAB. & CLIN. MED. **26**: 1672, 1941.
4. Eddy, B. E.: A Simplified Procedure for Detecting Cross Reactions in Diagnostic Antipneumococcic Serum, Pub. Health Rep. **59**: 1041-1045, 1944.

BOOK REVIEWS

Acetanilid: A Critical Bibliographic Review. By *Martin Gross*, M.D., Research Assistant (Assistant Professor), Laboratory of Applied Physiology, Yale University; with an introduction by *Howard W. Haggard*, M.D., Director, Laboratory of Applied Physiology, Yale University. Hillhouse Press, New Haven, 1946. Price $3.00. Cloth with 155 pages.

The content of 763 articles and textbook descriptions concerning acetanilid, a drug which is an active ingredient of many proprietary analgesic preparations, has been critically reviewed and presented in monograph form by Dr. Martin Gross. Support for this extensive bibliographic research came from the Institute for the Study of Analgesic and Sedative Drugs, which in turn derives its funds from contributions from the manufacturers of analgesic and sedative drugs. This is the first of a series of similar monographs soon to be published dealing with salicylates, phenacetin, bromides, and antipyrine.

The author has reviewed the widely scattered literature describing the first preparation of acetanilid in 1852 by Gerhart, the discovery of its antipyretic and analgesic properties by Cahn and Hepp in 1886, the ensuing years of its extensive exploitation for reducing fevers and the later development of therapeutic applications of its analgesic properties, the pharmacologic investigations of its mode of action and pathway of metabolic alteration in the body, and finally the beginning of search by David Lester (1943-1944) and others for the answers to problems described in the review. This summary serves, as Dr. Howard Haggard mentions in the foreword of the book, "to indicate the needs for more definite work on unestablished points and will lead to the avoidance of much unnecessary duplication of studies already made." Dr. Gross has included eight figures and twenty-four tables, many of which represent his own organization or graphic presentation of comparative data from the original sources.

The author's mode of handling controversial subjects is best exemplified by his treatment of the problem of the intermediary metabolism of the drug, a problem which he discusses in the light of twelve series of studies. Many widely quoted statements were found to be based on speculation. Concrete evidence was found available in the form of actual compounds identified and fragmentary evidence from studies of hypothetical substances forming methemoglobin.

In the chapter on pharmacology and toxicology, the mechanisms of and the factors affecting the formation of methemoglobin and of sulfhemoglobin are extensively discussed. It is made apparent that therapeutic doses of acetanilid produce little alteration of body functions other than those involved in analgesia and antipyresis. Many studies have been made with doses in the toxic range, and from these there has emerged no description of any characteristic effect, except for the appearance of cyanosis due to the formation of methemoglobin and sulfhemoglobin.

Despite many attempts several problems concerning acetanilid have been answered unsatisfactorily. Two problems are outstanding. No one has, as yet, adequately explained the mechanism by which it relieves pain, nor has the metabolic alteration of the drug been satisfactorily and definitely established.

Nearly one-half of the monograph is devoted to a résumé of 277 cases of acetanilid poisoning which have appeared in the literature. One-half of these cases occurred during the first ten years of its therapeutic use, a period of very extensive, often ill-directed and poorly controlled, clinical trial. At that time the drug was used mainly as an antipyretic and to a lesser extent as a local antiseptic dusting powder for abraded wounds and burns. In the last eighteen years, twenty-eight of twenty-nine cases of toxicity have resulted from the repeated and often prolonged administration of the drug. The only very characteristic sign of toxicity is cyanosis.

For those interested in the pharmacology and toxicology of analgesic drugs, this monograph will be a very useful guide and source of information.

HAROLD F. CHASE, M.D.

Early Ambulation and Related Procedures in Surgical Management. By *Daniel J. Leithauser*, M.D., F.A.C.S., Chief of Surgery, St. Joseph Mercy Hospital, Detroit, Mich. Charles C Thomas, Publisher, Springfield, Ill., 1946. Price $4.50. Cloth with 221 pages.

In this small monograph the history of early ambulation after operation is thoroughly but briefly discussed. The physiological justification for its use is carefully outlined, and the author's clinical results are tabulated in detail. Many photographs are presented illustrating the exact method recommended for the first termination of bed rest. The need for exercises and the importance of posture while in bed are also well emphasized and discussed.

By early ambulation the author means that the patient be made to walk within a few hours after operation, if possible, and always within the first twenty-four hours. He emphasizes this as a most important feature, particularly in order to avoid pulmonary complications and vascular accidents. The results achieved are described in considerable detail and include a great reduction in many postoperative complications and disability, a rapid restoration of the patient, and a great shortening in the length of hospital stay.

Over 2,000 operations in which early ambulation was employed form the basis of the author's clinical study. It should be noted, however, that the great majority of these operations were simple. There were only ten gastrectomies in the entire group. Although the period of hospital stay averaged just over two days in the case of appendectomy, patients who had had a gastrectomy remained fifteen days. One of the questions always raised in regard to early ambulation is the possibility that solid wound healing may be impaired thereby, particularly in the case of hernia operations. For this reason the data presented by the author on the recurrence rate following herniotomy are of special interest. In 235 patients operated upon, twelve, or 5.1 per cent, were found to have recurred "insofar as it can be determined by careful follow-up." This is not a high but still a substantial percentage. Yet it is not clear whether all of the patients operated upon were actually followed up. If not, obviously the twelve recurrences would add up to a much higher recurrence rate. The author has made a number of interesting technical observations, one of them of the effect of nylon sutures which he used in 100 patients; he found that in five of them difficulties followed, requiring removal of the sutures.

The monograph is well written and will prove of great interest to surgeons and others who have had no experience with early ambulation. It is no adverse criticism to state that the author is an enthusiast on the subject; indeed, enthusiasm is often needed to break down unjustified traditional resistance to change. On the other hand, it should be mentioned that the problem of postoperative care involves many factors other than the posture and movement of the patient. While a few of the related features have been discussed, the author did not sufficiently emphasize the obvious fact that early ambulation alone is not necessarily synonymous with a smooth postoperative convalescence. Moreover, there are certain contraindications to early rising after operation which are mentioned but insufficiently explained in merely thirteen lines of text. A lesser criticism might be the use of the term "laparotomy syndrome," inasmuch as laparotomy actually means an incision into the side of the patient. A more accurate term would have been celiotomy, but obviously this would not include such operations as herniotomy, thyroidectomy, and mastectomy.

The advantages of early termination of bed rest after operation have been repeatedly emphasized ever since they were first pointed out nearly fifty years ago, yet it is still not a universally applied principle in postoperative care. Surgeons, by clinging to past practices, frequently fail to achieve the maximum in efficient surgical convalescence. The present monograph should serve to challenge tradition and stimulate further interest in the subject, not only in surgical disease but in the therapy of any condition involving bed rest.

ROBERT ELMAN, M.D.